Some aspects of the microbiology of prepacked beef.

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SOME ASPECTS OF THE MICROBIOLOGY OF PREPACKED BEEF

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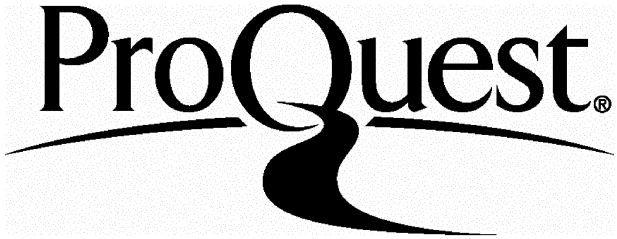

SUMMARY

The microbiology of prepacked beef steaks stored at chill temperatures (-1 or 4^{o}) was investigated. The changes in the composition of the microflora was determined by identification of isolates taken at intervals during storage. In the initial stages of the work the beef was subjectively assessed for acceptability by a panel of laboratory staff. Later, a colour measuring machine was also used.

Preliminary experiments confirmed that if the initial level of contamination was low and/or the temperature was -1^{o}, appreciable extensions in storage life were obtained. When steaks (packed in air with gas permeable film) were allowed to spoil, Pseudomonas was the dominant organism. No significant increase in storage life was found when a gas impermeable film was used, but under these conditions Microbacterium thermosphactum was the principal component of the flora.

Novel methods of preservation arose from studying the influence of various gaseous environments on the microbiological and colour characteristics of beef. A gas mixture containing 80% O_2 + 20% CO_2 gave marked increases in storage life by maintaining the beef pigment in the oxygenated state whilst simultaneously depressing the growth of the Gram negative aerobic spoilage flora. Further extensions in shelf life were achieved by combining gas packing and storage at -1^{o}. With gas packed beef stored at 4^{o} and -1^{o} the dominant microorganisms were M. thermosphactum and

Leuconostoc respectively.

The properties of a selection of the microbial groups isolated from beef i.e. Pseudomonas, M. thermosphactum, Acinetobacter, lactic acid bacteria, Micrococcus, Enterobacteriaceae and yeasts, were studied in detail. Based on these results the organisms were identified where possible to species level.

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GENERAL INTRODUCTION

Systematic study of the microbiology of muscle taken from domestic animals began at the turn of the century. It was soon established that the deterioration of stored muscle was usually due to the growth and activities of microorganisms (Glage, 1901; Tissier & Martelly, 1902). In this period refrigerated storage was the only commercial means of suppressing microbial growth and extending shelf-life. Thus Richardson & Scherubel (1908) noted little change in the chemical and histological characteristics and bacterial content of beef held at -9^{o}. Holding at these temperatures was used for prolonged storage of lamb, mutton and beef, particularly that intended for use in the manufacture of meat products such as sausages and pies. Nevertheless, the need to retain the fresh appearance of beef intended for retail to the consumer has prompted many detailed investigations of the microbiology of meats held at 0 to 5^{o}. In the period 1930 - 40 many of the underlying microbiological principles were recognized. For example, Haines & Smith (1933) and Haines (1937) noted that the time taken for the development of bacterial slime on stored beef was inversely related to the initial level of contamination, and that the Pseudomonas/Achromobacter complex was the principal component of the microbial association.

Prior to the early 1950's most fresh beef was only loosely wrapped when sold through small shops with quick turnover in stock. Thus the problem of microbial spoilage was confined mainly to stored carcasses. With the advent of supermarkets, particularly in the U.S.A.,

there was a rapid increase in the sale of prepacked cuts of meat. The latter are normally held in chilled cabinets for 3 to 4 days, and spoilage especially discolouration can be a major problem. It was shown that the microbiological principles pertaining to prepacked were essentially the same as those associated with unwrapped beef. Thus extension in shelf-life could be achieved by holding beef at temperatures just above its freezing point (c -2^{o}) and by improving the hygiene at all stages in the handling of meat (Ayres, 1955). Nevertheless, other means of preservation have been sought since at the present time the major cause of wastage is still due largely to microorganisms. Under laboratory conditions antibiotics (Ingram & Barnes, 1955) and irradiation, (Mossel, 1966) can retard microbial deterioration of meat. With the former case, practical application is restricted to poultry (U.S.A) and fish. * Irradiation as a means of preservation of foods has been prohibited in the U.K. (Statutory Instruments,1969).

In the search for novel methods for the preservation of prepacked meats, it was demonstrated that the gas permeability of the wrapping film influenced microbial development (Cavett, 1963). Thus low gas permeability film results in a build-up of CO_2 concentration which retards the growth of the normal spoilage flora (Ingram, 1962 a). However, under these conditions there is a concommitant depletion in O_2 and acceleration of undesirable changes in the beef pigment. Other methods such as the use of the bacteriacidal

* This situation is under review, and it seems likely that use of antibiotics will be limited even more strictly.

agent O_3 has attracted attention (Ingram & Barnes, 1954) but extension of storage life of fresh meats is small unless high concentrations of O_3 are used. With low concentrations (10ppm) the extensions are at best only a few days (Kaess & Weidemann, 1968).

When beef is prepacked it can be considered an ecological system useful for studying the interaction of microorganisms with their environment and vice versa. In the present studies particular attention was given to the interrelationships of internal atmosphere, microflora and other characteristics (e.g. colour) of refrigerated prepacked beef steaks. The practical aims of the work were to devise storage conditions, particularly with respect to the gaseous environment, whereby microbial growth would be depressed and an acceptable red colour of the beef maintained.

The thesis is divided into two sections. The first (Section A) deals with the influence of various factors on the microbiological and other characteristics of stored beef and how the interaction of these can determine which microbial types become dominant. Section B is concerned with the properties and possible identity of the major microbial groups isolated during the investigations.

Each section also contains a discussion of the literature in relation to the results reported here.

S E C T I O N A

SOME FACTORS INFLUENCING MICROBIAL DEVELOPMENT ON PREPACKED BEEF

INTRODUCTION

Prepacked beef was considered an ecological system, and particular attention given to the interrelationships of various gas mixtures (O_2, CO_2, N_2), microflora and organoleptic properties, of beef steaks stored at -1 or 4^{o}.

MATERIALS AND METHODS

Meat

Source

The meat used in all experiments was obtained, unless otherwise stated, from a local butcher who slaughtered and dressed animals in an abbatoir attached to the premises. The animals (Black Poll) were rested in a paddock for 3 h and then led to a pen where they were stunned by a captive bolt, pithed and bled in a conventional manner. Sides of beef were held at c 4^{o} for 10–12 d.

Preparation of samples

Thick flanks of beef (transverse sections of the following muscles, vastus group, sartorius, rectus femoris, tensor fascia latae) were removed by the butcher. These were immediately taken to the laboratory and slices (weight, c 100 g; thickness, c 2 cm) prepared.

Packaging

The steaks were wrapped in transparent film and the packages heat-sealed ("Pyramid" hand sealer, A.H. Bland Ltd., London). Two types of film were used, one having high gas permeability ("Meatwrap", British Visqueen Ltd., London; ethylene vinyl acetate co-polymer; 100 gauge; 0_2 permeability 4.9 $cc/cm^2/sec/cm\ Hg/cm \times 10^{-10}$; H_2O vapour permeability 1.3 $g/m^2/24$ h) the other a low gas permeability (300 gauge MXDTA laminate polyethylene; 0_2 permeability c 1 $cc/cm^2/sec/cm\ Hg/cm \times 10^{-14}$, H_2O vapour permeability 6 $g/m^2/24$ h). The wrapped steaks were numbered and stored (single layers) in a refrigerator.

Gas packaging

Steaks were placed in bags (12 x 16 cm), a tube was inserted and the contents flushed for c 10 sec with c 250 ml of an appropriate gas mixture (details in text). The latter were obtained from British

Oxygen Company Ltd., London. To prevent fractionation of the gases, the cylinders were held horizontally for a minimum of 48 h before use. The composition of the gas mixtures were determined routinely by means of Fisher Gas Partitioner (for details of method, see below).

Sampling

Random number tables were used to ensure unbiased selection when removing steaks from the refrigerator.

Gas analysis

The composition of the atmosphere in the packs was determined by gas/solid chromatography. A sample (0.5 ml) was injected into a Fisher Gas Partitioner Model 25 M (Shandon Ltd., London), helium (flow rate 50 ml/min) was used as the carrier-gas. Percentages of oxygen, nitrogen and carbon dioxide were calculated from peak heights on a recording potentiometer.

Colour measurement

A "Colourcord" tristimulus colourmeter (Joyce-Loebel Ltd., London) was used. A piece of the film was removed from the upper surface of the packaged steak, and a metal plate with a 1" diameter hole was placed on the steak so that the latter was aligned with the surface of the exposed meat. The colour was measured and the plate realigned onto another part of the steak and a further reading taken. This procedure was repeated as many times as possible (minimum of 4 readings) on every sample.

Subjective assessment

The meat samples were assessed by 4 members of the laboratory staff. Particular attention was given to the colour of the beef and whether or not odours and slime were present. The meat was considered to be unacceptable when any one of these was judged to be unsatisfactory.

Microbiological analysis

Using aseptic techniques, the steaks were cut into 2 cm cubes. A random sample of the cubes (c 30 g) was placed in a homogenizer (Atomix, M.S.E. Ltd.) and sufficient 0.1% (w/v) peptone water was added to give a tenfold dilution. The meat was blended for 2 min at 2000 rpm. A 1 ml sample of the homogenate was removed and tenfold serial dilutions prepared in the diluent noted previously.

Viable count. Known volumes (0.1 ml) of appropriate dilutions were spread on the surface of Oxoid Plate Count Agar (PCA). Duplicate plates were prepared from each dilution. Prior to inoculation, the surface of the agar was dried by holding for 1 h at 37°. Incubation was at 20° for 3 d.

Yeasts. Potato Glucose Agar, acidified (pH 3.5) with citric acid, was used for the recovery of yeasts. The medium was incubated for 5 d at 25°.

Lactic acid bacteria. The medium described by Rogosa, Mitchell & Wiseman (1951) was used. It was inoculated as noted above, overlayed with about 5 ml of the same medium and incubated for 5 d at 20°.

Characterization of microorganisms

To ensure a random selection of colonies which had developed on PCA the method described by Baird-Parker (1962) was used. The bottom of the Petri dish was marked to give 8 equal segments. These were numbered thus: 1, 7, 6, 3, 2, 8, 5, 4. All the colonies were picked from segments 1, 2 et seq - until the required number of isolates had been recovered. The colonies were transferred to slopes of Difco Heart Infusion Agar (HIA) and, after incubation for 3 d at 20°, they were subcultured on PCA until pure and maintained (4°) on HIA slopes. Details for the characterization of strains are given in Section B (page 63).

RESULTS

A survey of various techniques for the recovery of viable bacteria from raw meat was carried out (Mr. A.W. Knight pers.comm.). The performance of following media was assessed; Lab Lemco Glucose Agar, APT Agar* (Evans & Niven, 1951), Nutrient Agar (Oxoid), Plate Count Agar (Oxoid), Yeast Extract Glucose Agar, Heart Infusion Agar (Difco) and Blood Agar (Oxoid). In addition, spread and pour plate techniques were compared. The results showed that APT, Nutrient and Lab Lemco Glucose agar gave significantly (P= 0.05) lower counts than the other media tested. Moreover the spread plate method gave significantly (P=0.01) higher recovery of bacteria than pour plate techniques. Spread plate methods also allowed easier isolation and subculture of colonies. These findings are in agreement with those of the International Organisation for Standardization (1967) who investigated various media and methods for the total aerobic bacterial count from meat and meat products. They recommended that maceration of the sample, together with spread plate techniques, be used for the recovery of microorganisms. These methods were used throughout the present study.

Influence of size of initial infection

At the outset of this investigation attempts were made to repeat the observations of previous workers. In particular the effect of the

* All Purpose Tryptone

level of the initial infection on the storage life of beef and the gross composition of the microbial association present at the time of spoilage were examined. The available evidence indicates that the majority of the causative organisms of spoilage of chilled beef are acquired post mortem (Ayres, 1955) and that they are located at or near the surface (Moran, 1935). To obtain steaks having minimal contamination, a surface layer (c 2 cm) of the flank was removed under aseptic conditions. Portions taken from the underlying muscle tissue harboured c 1.0×10^1 - 1.0×10^3 bacteria/g, whereas those taken from untrimmed flanks gave counts of up to 1.0×10^6/g (Fig.1). Slices of meat were wrapped with the highly gas permeable film ("Meatwrap") and stored at 4^o. At intervals during storage, randomly selected steaks were subjectively assessed and the extent of microbial contamination determined. The results are summarized in Fig. 1.

When care had been taken to minimize the initial infection, the meat was considered to be acceptable until the 6-7th d at which time the total viable count was 1.0 - 10.0 x 10^7 bacteria/g. Populations of this magnitude were present by the 3-4th d of storage when no precautions had been taken to reduce the starting level of contamination. In all instances discolouration and/or off-odours were the first manifestations of spoilage noted by the laboratory assessment panel. These characteristics were associated with a count of c 10^7 organisms/g; slime was observed when the number of bacteria approached 10^8/g. Pure isolates obtained from randomly selected colonies on PCA were characterized in sufficient detail to assign to families or genera. It was found that with different

initial levels of contamination, the composition of the flora when the meat had spoiled was similar in all cases, Pseudomonas was dominant and comprised upwards of 80% of the flora.

Influence of temperature

The behaviour of microorganisms on steaks (wrapped with gas permeable film) stored at -1.5, 1 or 4^{o}was examined. Laboratory personnel assessed the quality of the meat, and changes in the size and gross composition of the microflora were determined by the methods noted previously. The temperatures at selected sites within the refrigerator were determined. Thermocouples (40 gauge, copper/constantin) were inserted into slices of wrapped beef. Four such packs were prepared and placed at various positions in the cabinet. The thermocouples were connected to a Micrograph BD1 recorder with Channel Selector BA1 - tolerance $\pm$ 0.01^{o} (Kipp & Zonen, Delft, Holland). Recordings were made for upwards of 3 d. It was found that the maximum temperature variation at any given position was $\pm$ 0.2^{o}, and the maximum difference between any two positions was 0.5^{o}. Values of this order were obtained when the cabinet was held at the three settings(-1.5, 1, or 4^{o}). Moreover it was noted that no significant temperature change occurred when the cabinet was opened to remove samples.

The development of the microbial populations on meat held at -1.5,1 or 4^{o} is shown in Fig.2. With samples held at 4^{o} populations of 10^{8}/g were attained by the 8th d of storage at which time all the samples were judged to have spoiled. In contrast, with storage at 1^{o} (Fig.2a) the rate of microbial growth was appreciably slower.

On the 8th d the count was $\underline{c}$ 10^5/g and the meat was judged to be acceptable. A greater retardation of growth was found in samples stored at -1.5°(Fig. 2c). At this temperature the total count apparently did not increase during the first week of storage and the steaks were still considered to be acceptable on the 12th d. The differences in rate of microbial growth observed at each temperature did not influence the composition of the spoilage flora. Thus analysis of the changes in the flora showed that Pseudomonas predominated at all three temperatures and comprised $\rangle$ 80% of the flora on the final sampling day.

Influence of film permeability

Changes in the size and composition of the flora on steaks (stored at 4°), packed with either the gas permeable film ("Meatwrap") or the impermeable film (MXDTA/polythene) were examined. The Fisher gas analyzer was used to monitor changes in the composition of the atmosphere surrounding the meat.

The results obtained are summarized in Fig. 3. When the atmosphere in the gas permeable packs was analyzed 4 h after packaging, it was found that the CO_2 content was 4% (v/v). It is unlikely that this increase was due to bacterial activity; it probably arose from dissolved CO_2 present in the meat (Urbin & Wilson, 1961). On the 4th d the concentration of CO_2 was 1% (v/v) suggesting that, during the first few days of storage, loss of CO_2 by diffusion through the film was greater than that produced by the meat and/or microorganisms. Thereafter a progressive increase in CO_2 was noted and on the 23rd d it comprised $\underline{c}$ 10% (v/v) of the atmosphere. On the other hand there

was a gradual reduction in the concentration of O_2, there being 2% (v/v) in packs examined on the 23rd d of storage. With meat packed in impermeable film, there was a rapid decrease in O_2, and the rate of build-up of CO_2 was appreciably faster than that noted previously. Thus it accounted for 36% (v/v) of the atmosphere in packs examined on the 23rd d.

The rate and extent of microbial development (Fig. 3b) on steaks contained in gas permeable film were essentially the same as that discussed previously (Fig.1). With the impermeable film a slower rate of development and smaller final population were observed (Fig. 3b). Gross changes in the composition of the flora were followed by examination of Gram-stained films (1280 films examined). These were prepared from randomly selected colonies on PCA. The composition of the final populations were markedly different (Fig. 3c). In keeping with previous experience, Gram negative organisms became dominant in meat packed in permeable film. They were a minor component of the flora developing on meat wrapped with impermeable film. In this case Gram positive rod-shaped organisms were predominant.

In a repeat of these experiments attempts were made to identify the dominant organisms in the associations. Changes in the composition of the atmosphere within the packs and the size of the populations (Fig. 4) were essentially the same as those discussed above (Fig. 3). Twenty isolates taken from PCA plates prepared from each sample of meat were identified according to the scheme set out in Table 2. The predominant Gram positive rods in gas

impermeable packs were identified with Microbacterium thermosphactum as defined by McLean & Sulzbacher (1953). It is noteworthy that these organisms caused changes in the meat different from those occurring in that held in permeable film. With the latter (Gram negative organisms predominating) an off-odour together with slime were noted on the 10th d at which time the populations were of the order of 10^8/g. Slime did not develop on meat packed with impermeable film even though its surface harboured populations of 10^8/g. Nevertheless, off-odours were noted; a musty odour was apparent on the 10th d of storage and a slightly sour odour on the 19th d.

The colour changes of meat in contact with impermeable film were similar to those described by Brooks (1929) and Kraft & Ayres (1952) and they can be interpreted in terms of the oxygenation/reduction of myoglobin:

$$\underset{Fe^{++}}{\text{(bright red) oxymyoglobin}} \underset{+O_2}{\overset{-O_2}{\rightleftharpoons}} \underset{Fe^{++}}{\text{myoglobin}} \xrightarrow[\text{reduction}]{\text{oxidation at low } O_2 \text{ tension}} \underset{Fe^{+++}}{\text{(brown) metmyoglobin}}$$

Three distinct colour regions were evident (Fig. 5). Thus there was a central purplish red area (myoglobin) bounded by a brown ring (metmyoglobin), the outer edges being bright red (oxymyoglobin). These bands of pigmentation were not a feature of beef wrapped with permeable film. They illustrate the general effects of O_2 on beef pigment. Thus at the edge of the steak there was sufficient O_2 to maintain the pigment in the oxygenated state. Moving inwards, the

O_2 tension dropped and at c 2–3 mm it is optimum for the formation of brown metmyoglobin (Brooks, 1929). In the central region the occurrence of myoglobin was almost certainly associated with a very low O_2 tension.

Although the use of permeable film resulted in meat being exposed to an atmosphere which became progressively depleted in O_2 but enriched with CO_2, the overall rate and extent of microbial growth and the composition of the spoilage flora was essentially similar to that reported above (Fig. 1). With the gas impermeable film there was a more marked build-up of CO_2 in the atmosphere. The inhibitory effects of CO_2 on microorganisms has long been known, and it has been used for the preservation of meat, vegetables, etc. In the present study, also, a small increase in shelf-life was found in impermeable packs. Carbon dioxide has been considered to have a selective action: Gardner & Carson (1967) concluded that the development of M. thermosphactum and lactic acid bacteria was due to the organisms tolerance of CO_2. Such an interpretation would appear to be in accord with the observations made in this study. Even though the increase in CO_2 led to a slight extension in the storage life of steaks held in impermeable film , concomitant undesirable changes in the meat colour (Fig. 5) would appear to preclude the commercial use of such wrapping material, since beef colour is probably the most important single criterion for consumer acceptance (Niven, 1951; Landrock & Wallace, 1955; Urbin & Wilson, 1958). It was for this reason that attempts were made to find a mixture of gases which would hinder microbial development whilst at

the same time maintaining the meat pigment in the desired bright red (oxymyoglobin) state.

Influence of selected gas mixtures

In these studies the effects of various combinations of O_2, CO_2 and N_2 on the microbial development and other characteristics of fresh beef were determined.

From the results reported by others (Brooks, 1933; Moran, 1935; Ogilvy & Ayres, 1951) it was realized that the initial concentration of CO_2 should not exceed $\underline{c}$ 20% (v/v) if the meat pigment was to be maintained in the oxymyoglobin state. In the following section results are given from tests in which beef was packed in gas mixtures none of which contained $>$ 20% CO_2 (v/v). Steaks were placed in envelopes of MXDTA/polythene, flushed with an appropriate gas mixture and then heat sealed. This inflated the pouch so that the upper surface of the meat was not in contact with the film. The gas mixtures were supplied by British Oxygen Company Ltd. and their composition checked. The steaks were stored at 4^{o}, assessed and examined microbiologically as described above. Fig. 6 contains a summary of the results obtained from examination of beef held in an atmosphere consisting at the outset of (% v/v) CO_2 (20), O_2 (20), N_2 (60) or in gas permeable pouches ("Meatwrap") containing air. In the latter case the sequence of changes in O_2 and CO_2 content of the atmosphere (Fig.6a) was as described earlier (Fig.4). The proportion of the individual gases changed during storage of meat in the inflated pouches; the concentration of CO_2 increased to 22% (v/v) and that of O_2 decreased

to 4% (v/v).

When the rate of growth of organisms on the beef was compared (Fig.6b) it was seen that it was markedly reduced when the atmosphere contained 20% CO_2 at the beginning of the experiment. Thus at 4 d a tenfold difference in total bacterial count was noted. This difference persisted until the final sampling day. It would appear that the smaller populations did not arise through competition between microorganisms. Thus it was noted that gas packing apparently did not encourage the growth of lactic acid bacteria; the population attained by these organisms was similar in both gas and air packed beef. Characterization of isolates recovered on PCA showed that Gram negative organisms were again predominant in air packed samples whereas M. thermosphactum soon became the principal contaminant in steaks held in the gas mixture.

Although the atmosphere consisting initially of (% v/v) CO_2 (20), O_2 (20), N_2 (60) delayed microbial deterioration of the beef, as judged by laboratory personnel, it did not prevent undesirable change in the meat pigment. Brown discolouration (metmyoglobin) was observed at about the same time in both air and gas packed samples. This was again attributed to reduction of O_2 tension below the level necessary to conserve the oxymyoglobin.

In an attempt to maintain an adequate O_2 tension, the following gas mixture (% v/v) CO_2 (20), O_2 (80) * was used to fill meat packs

* The use of this gas mixture in the preservation of meats and other foods is included in British Patent Application No.16133/68 (C.M.Davidson & D.L. Georgala, Unilever Ltd.)

as described earlier. Storage was at 4^{o}. In addition to microbiological studies and subjective assessment of quality, the colour of steaks was measured. With the impermeable film the concentration of CO_2 increased slowly during storage and was 29% (v/v) by the 25th d (Fig.7a). In these packs there was a reduction in O_2 from the initial level of 77% to 72% (v/v). The total numbers of bacteria recovered from gas-packed beef was always less (upwards of tenfold) than comparable air-packed steaks wrapped in permeable film (Fig. 7b). A reduction in count was noted in the gas packs at the 4th d. This may have been due to pack-to-pack variation but it is possible that the gas mixture may have been toxic to some members of the initial flora. In addition rates of growth of yeasts and lactic acid bacteria appeared to be faster in air packs as compared to gas-packs.

A detailed study of isolates taken from PCA (Fig.7c) have a picture similar to those found previously (Fig. 4,6). Pseudomonads became dominant in air packed samples whereas their numbers (calculated on the basis of their percentage contribution to the flora) in gas packed samples remained constant ($\underline{c}$ 10^6/g) after 12 d. With samples stored in the gas mixture M. thermosphactum achieved dominance (60%) by the 4th d, and their numbers increased steadily until the final sampling day when they were present at levels of $\underline{c}$ 10^8/g and comprised 99% of the flora.

The mixture of (% v/v) O_2 (80) : CO_2 (20) delayed undesirable changes in the meat pigment, the steaks having an acceptable red

colour on the 12th d of storage. Moreover, evidence of microbial deterioration (off-odours or slime) were not noted at this time. Although it was assumed that the delay in the time taken for spoilage to occur was due principally to the high concentration of CO_2, it was considered necessary to check the influence of the elevated O_2 tension. Thus the effects of gas-packing in a mixture (% v/v) O_2 (80) : N_2 (20) were determined (Fig. 8). Although the meat held under these conditions retained an acceptable colour longer than the control meat, it supported large populations of Gram negative organisms which produced slime by the 8th d. It therefore seems reasonable to assume that high O_2 tensions do not have any appreciable effect on the growth of the normal aerobic spoilage flora, a conclusion which receives support from the work of Shaw & Nicol (1969). These authors found that the growth of a Pseudomonas on muscle slices was unaffected by concentrations of O_2 up to 100% (v/v).

Influence of gas-packing (80% O_2 : 20% CO_2) and storage at -1°

In an attempt to further extend the storage life of prepacked beef, the effects of low temperature in combination with storage under 80% O_2 : 20% CO_2 were examined. Randomly selected steaks were packed in permeable or impermeable films and, after the latter had been flushed with the gas mixture, they were stored at -1 or 4°. It was found that there was no significant difference in the changes in the proportions of CO_2 and O_2 in the atmospheres of packs held at these temperatures. Likewise the microbiological changes (Fig. 9) occurring on beef either in impermeable or permeable films and held at 4° were the same as those noted above. As would be expected there

was a slower development of the association with the air-packed beef stored at -1^{o}, the period of significant growth extending over 11d. Thereafter growth was slower and the population (5×10^{8}/g) was about tenfold less than that occurring at 4^{o}. Erratic results were obtained (Fig. $9b_2$) from an examination of the size of the populations on gas-packed samples held at -1^{o}. Nevertheless the data indicate that the bacteria grew albeit more slowly than with any of the other systems included in this study. This was reflected by the populations (10^{7}/g) present at 28 d. In addition to differences in population size, it was noted from a detailed examination of the composition of the flora, that storage of meat in $O_2 + CO_2$ at -1^{o} selected organisms different from those found previously. On PCA there was a marked increase in the incidence of leuconostocs on and after 16 d. It will be seen (Fig. $9c_2$) that these organisms formed at least 90% of the flora on the final sampling day. Only a small proportion of these organisms were tolerant of acetate at pH 5.4; the counts on Rogosa agar were about 1/1000th those of the catalase negative colonies on PCA. These organisms were invariably recovered from beef obtained from several suppliers. It can be deduced, therefore, that they form a normal component of the flora of beef. However, under normal storage conditions they do not achieve numbers sufficient to allow their isolation on media used routinely in the bacteriological examination of meat.

Colour changes in prepacked beef

It has been repeatedly stressed (Landrock & Wallace, 1955; Pirko & Ayres, 1955; Brown & Tappel, 1958; Urbin & Wilson, 1958)

that a desirable red colour in fresh meat, especially beef, is probably the most important factor for acceptance as judged by the

consumer. One of the aims of the present work was to investigate novel methods of preservation which might in addition to controlling microbial deterioration, conserve the desirable colour of prepacked beef steaks. The effects of different storage conditions on the characteristics of beef were subjectively assessed by laboratory staff. In most instances their assessments were in agreement, but occasionally quite different scorings were obtained from one individual to another. To remove bias, due to subjective assessment, it was necessary to determine accurately the beef colour. This was done with a colour measuring machine ("Colourcord" Tristimulus colourmeter, Joyce Loebel). Three independent properties of the colour were determined : the dominant wavelength, the purity and the luminance. The dominant wavelength is the wavelength of spectrally pure energy that if mixed with white would match the colour. The purity is the ratio of the spectral to the sum of the spectral and white components. The luminance, once called the photometric brightness, is the luminance intensity of reflected light. The first two properties, dominant wavelength and purity together are termed the chromaticity of a colour. The chromaticity can also be expressed as a point, co-ordinate $(x_1\ y_1)$, on a chromaticity diagram. The "Colourcord" measures colours by recording the percentage of red light(X) blue light (Z) and green light (Y) reflected from the sample. These readings are then converted to chromaticity co-ordinates $(x_1 y_1)$ thus:-

$$x = \frac{X}{X + Y + Z} \qquad y = \frac{Y}{X + Y + Z}$$

The third co-ordinate, Y = luminance.

The above measurements were confined to experiments on the effects of gas-packing on the storage life of beef steaks. Fig.10, 11, 12 show the results of colour measurements on beef packed under 80% O_2 20% CO_2, and stored at 4^{o}. The area on the chromaticity diagram where the meat chromaticity lie is illustrated in Fig. 10. Fig.11 is an expanded view of this area, lines of dominant wavelength and purity together with storage time are included. The data are illustrated in simplified form in Fig. 12. On day 0 the gas-packed were visually a brighter red than the air-packed steaks. This was probably due to the increased oxygen tension in the gas-packs resulting in the formation of more oxymyoglobin and is reflected in the lower dominant wavelength (oxymyoglobin being a lighter more yellow red than the characteristic purplish red of myoglobin). The most significant colour change during storage of both gas and air-packed samples was in purity. It will be seen (Fig. 12a) that the colour purity of the meat in both types of pack fell steadily but at a faster rate with the air-packed meat. Thus at 12 d purity readings on gas-packed samples were $\underline{c}$ 40%, whereas with air-packed samples the purity had fallen to 30% by 7 d. Changes in dominant wavelengths were also markedly different (Fig. 12b). Thus the gas-packed samples remained at a constant value ($\underline{c}$ 594 mμ) whereas the air-packed meat decreased ($\underline{c}$ 590 mμ). It was notable that these results correlated well with the subjective assessments. The panel agreed that the gas-packed beef retained an acceptable red colour for a significantly longer period. Further prolongation of colour shelf life was achieved when beef was packed in 80% O_2 : 20% CO_2 and stored at -1^{o}. Again purity and dominant wavelength were the parameters which have the best indication of comparative changes (Fig. 13). With the beef

held in the gas mixture, the purity reading was maintained at about 35% until the 28th d, whereas the purity of air-packed samples was $\underline{c}$ 30% after 7 d. The results of all colour measurements were summarised as a discrimination diagram (Fig.14); luminance is plotted against chromaticity co-ordinate x_1 and acceptable and unacceptable samples (by panel assessment) are shown. A value of $x \rangle .420$ was characteristic of acceptable samples, and this may have practical application for predicting potential "colour life" of beef.

DISCUSSION

Source of infection

It is generally agreed that the microorganisms present on fresh meat are acquired during the slaughtering of the animal and butchering of the carcass. Although large numbers of organisms are present on the skin and in the intestinal tract of animals, constitutive and inducible defences provide a barrier to microbial invasion. The actual effectiveness of such barriers is not easily assessed due to technical difficulties associated with the aseptic sampling of tissues. It is for this reason that some of the early examinations of organs from healthy animals have to be treated with caution. The consensus of opinion suggests that small numbers of organisms enter the tissues of healthy animals. It is suspected that their presence is not due to active invasion but rather to their passive transport across the defences, a process for which the term translocation has been proposed (Wolochow, Hilderbrand & Lamanna, 1966). Such organisms, being mesophilic, are unlikely to participate in the spoilage of chilled beef, providing cooling of the carcass is sufficiently rapid. With the whale, for example, its mass precludes rapid chilling and the muscle is spoiled by mesophilic *Clostridium* derived from the gut (Ingram, 1962b).

The principal depots of infection of dead flesh are probably the hides of cattle (Empey & Scott, 1939), fleece of sheep (Patterson, 1967), feet and feathers of poultry (Barnes, 1960) and the gills and integuments of fish (Shewan, 1961). Under commercial conditions the main vehicles for the transport of organisms to freshly cut surfaces can be considered to be the utensils, working surfaces and hands and

clothing of the operators.

Microbial associations on stored beef

From a consideration of the depots of infection it is not surprising that freshly butchered meat harbours a heterogeneous flora. Thus representatives of about 20 bacterial genera, 9 moulds and 3 yeasts have been isolated (Ayres, 1951). In the present study members of the following groups were recovered from refrigerated beef: Pseudomonas, Acinetobacter, Microbacterium, Lactobacillus, Leuconostoc, micrococci, Enterobacteriacae and yeasts.

Beijerinck (1908) was probably the first to recognize that a specific type of deterioration usually occurs in any food held under given conditions and that relatively few of the initial contaminants contribute significantly to the spoilage flora. It follows, therefore, that appropriate laboratory methods must be employed if the development of the association in a particular food is to be meaningfully determined. With some of the early work on refrigerated meats for example, incubation at 37^{o} was used (Weiznirl, 1924; Hoffstadt, 1924). The types of organisms recovered (e.g. Sarcina and Proteus) differ significantly from those (Pseudomonas/Acinetobacter etc.) of later workers and in this study where media was held at 4 or 20^{o}. The early work does, however, demonstrate that mesophilic organisms are not rapidly killed by chilling, (Ingram, 1951) they can remain quiescent and are overgrown by psychrophiles.

Haines (1933a) and Empey & Scott (1939) were perhaps the first to appreciate the full role of aerobic Gram negative rods in the deterioration of chilled beef. This finding has been common to most

subsequent investigations. The early investigators identified the majority of their isolates with Achromobacter and found that the development of surface slime was due to the confluent growth of these organisms. Due to revision in the taxonomy of the pseudomonads, most of these organisms would now be placed in the Pseudomonas. Thus almost all of 129 strains originally isolated by Empey & Scott (1939) have been subsequently identified with Pseudomonas (Brown & Wiedemann, 1958).

Ayres (1951) reviewed the bacteriological aspects of meat spoilage. Subsequent investigations into the microbiology of both unwrapped and prepacked beef have confirmed the findings of the early workers. Kirsch, Berry, Baldwin & Foster (1952) followed the changes in the flora of minced beef stored at 0 - 2°. An examination of 610 isolates indicated that non-pigmented pseudomonads and Achromobacter (Acinetobacter) became dominant in all samples. In one of the samples, organisms identified with lactic acid bacteria contributed c 35% of the flora after 15 d. This observation is questionable since it is probable that with incubation at 30° a false negative catalase reaction was obtained. It is conceivable that these isolates would now be identified with M. thermosphactum, an organism that can give a negative catalase reaction when grown at 30°(Davidson, Mobbs & Stubbs, 1968). In another sample, Kirsch and his collaborators isolated large numbers of Proteus. The identification of these organisms was based on insufficient evidence; Gram-stain, urease reaction and a putrid odour from the beef, therefore the assumption that they were Proteus is debatable. In an investigation on beef hamburgers

(Rogers & McCleskey, 1957) the initial infection was dominated by Gram negative rods and micrococci. During storage at 7°, members of the Pseudomonas/Achromobacter (Acinetobacter)complex comprised 84% of the flora at 14 d. At this time Bacillus (6%), Microbacterium (5%) and micrococci (5%) were also isolated. Since the population increased almost a million fold during storage, it can be deduced that there was active proliferation of these groups. Although it is known that some micrococci and Bacillus can grow at temperatures around 10°, it seems unusual that they would still form such significant proportions of the association after 2 weeks storage. There is a possibility, therefore, that contamination may have occurred during sampling.

The microbiological changes on unwrapped beef, held at 0-2° in a moist atmosphere, were studied by Wolin, Evans & Niven (1957). They showed that Pseudomonas and Gram positive rods identified with M. thermosphactum were important contaminants of freshly prepared samples. When the beef was stored until spoilage was evident, 90% of the flora consisted of pseudomonads. In a more recent study (Ayres, 1960) the microflora developing on slices of beef held at various temperatures (0-20°) and packed in MSAD cellophane was examined. The initial flora consisted of several genera, but only Pseudomonas, Achromobacter, Flavobacterium, Micrococcus, Microbacterium and Penicillium were recovered regularly. With samples held at 10° or less, pseudomonads were responsible for slime production. At 15° or above, Pseudomonas and Micrococcus were present in roughly the same proportions. The aerobic flora has also been studied by Gardner (1965), who used selective media

to monitor changes in the numbers of Pseudomonas-Achromobacter, Micrococcus, Streptococcus, coli-aerogenes bacteria, yeasts and moulds in ground beef stored at 15, 9 and 4°. At the higher temperature, Group I and Group II Pseudomonas (Shewan, Hobbs & Hodgkiss, 1960) together with the coli-aerogenes organisms were prevalent at 5-6 d storage. With storage at 9 and 4°, the latter were not detected on total count media, and Pseudomonas became predominant as the beef deteriorated.

In the present investigations, the results obtained with beef (stored at -1 or 4° and packed with permeable film) are in accord with those discussed above. Although the initial flora was heterogeneous, Pseudomonas invariably outgrew the others and could form upwards of 90% of the flora of spoiled steaks. M.thermosphactum and, less frequently, Acinetobacter were the only other organisms which contributed significantly. It has been established that the microflora developing on other chilled meats and fish stored under "aerobic" conditions is also dominated by the Pseudomonas and/or Acinetobacter. This has been demonstrated with poultry (Ayres, Ogilvy & Stewart, 1950; Nagel, Simpson, Vaughn, Ng & Stewart, 1960; Thornley, Ingram & Barnes, 1960; Barnes & Thornley, 1966), pork (Gardner, Carson & Patton, 1967) and fish (Shewan, 1961).

Some unusual associations have been noted in the literature. Thus Mallmann (1932) found that organisms resembling Bacillus mesentericus were responsible for slime on ducklings stored at 10°. However, it should be pointed out that during processing, the ducks

were scalded (60^{o}), a process which would be expected to kill Pseudomonas etc. but permit the survival of spore-formers. Indeed, the causative organisms were isolated in large numbers from the scalding tank and, therefore, it is not surprising that they became the dominant type during chilling and storage. Of interest too, is the association found on lamb chops (Barlow & Kitchell, 1966). With a gas permeable film (Cryovac XL), the spoilage flora was composed mainly of M. thermosphactum. However, as the authors noted, the high O_2 permeability of this film is dependent on contact with the moist surface of the meat. In their experiments this was not always the case. When dry, the Cryovac XL behaves as a film with relatively low gas permeability. Under such conditions the predominance of M. thermosphactum is not unexpected and is in accord with the results obtained in the present work. Thus it was shown (Fig. 4, 7) that with beef packed in air or certain gas mixtures e.g. 80% O_2 : 20% CO_2 and stored at 4^{o}, M. thermosphactum could reach populations representing 90% of the flora. There are, however, associations whose origins cannot be easily explained. Microbiological changes on beef, lamb and pork packed with films of different permeabilities were followed by Halleck, Ball & Stier (1958). With beef packed with impermeable film it was claimed that non-pigmented Pseudomonas and lactobacilli predominated during the first two weeks storage at 1.1 - 3.3^{o}. The organisms designated lactobacilli were unusual in that they grew well on media lacking carbohydrate, and although the available data are incomplete it would seem reasonable that these organisms would now be identified with M.thermosphactum. During the latter part of the storage period, the flora consisted mainly of organisms identified with Ps. fluorescens which were present in numbers equivalent to c 90%

of the total count. This finding is difficult to explain in the light of the present and other studies where it has been shown that the gaseous exchanges, particularly build-up in CO_2, in impermeable packs caused a marked depression in growth of Pseudomonas. Halleck et al. (1958) also estimated the proportion of oxidase positive bacteria on stored meats. With stored beef, for example, it was reported that the Ps. fluorescens count was tenfold greater than the oxidase positive count. This result is questionable since this species normally gives a strong positive in the oxidase test. The effects of film permeability on meat microflora were also studied by Jaye, Kittaka & Ordal (1962). Samples of ground beef were packed either in saran tubing (gas impermeable) or MSAD cellophane (gas permeable) and stored at -1^{o} or 3.5^{o}. With saran packed samples stored at 3.5^{o}, heterofermentative lactobacilli and Leuconostoc mesenteroides could comprise 50% of the total count. Numbers of aerobic spoilage organisms (indicated by proportion of pigmented Pseudomonas) increased only marginally. Although a similar rate of increase in numbers of lactic acid bacteria was noted in cellophane-packed beef, this increase represented only a small fraction of the total count. A more recent study (Gardner et al., 1967) was concerned with effect of films on the bacteriological aspects of chilled pork. With impermeable packs stored at 2^{o} for 14 days, lactobacilli and M. thermosphactum comprised 30 and 18% of the flora respectively. In samples wrapped with permeable film, these percentages were appreciably lower, 11 and 4%, and the Pseudomonas/Acinetobacter complex were present in numbers equivalent to 83%. The authors' conclusion that the build-up of CO_2 in impermeable packs inhibits the Pseudomonas and Acinetobacter receives support from the present work (Fig. 4).

Other storage conditions or treatments have also been shown to allow M. thermosphactum and/or lactic acid bacteria to form significant proportions, or even become dominant in the flora of meats, meat products and fish. Thus, when beef was stored (59 d) under N_2 (Weidemann, 1965), microbacteria (probably M. thermosphactum) accounted for c 95% of the flora of samples held at 0^o. Similarly with irradiated beef (Wolin, Evans & Niven, 1957) and chicken (Thornley, 1957), vacuum-packed fish (Licciardello, Ronsivalli & Slavin, 1967) sausages (Dowdell & Board, 1968) organism identified with or closely resembling M. thermosphactum have been shown to outgrow other microbial types as the product spoils. In other studies (e.g. Noskova & Pek, 1963) lactic acid bacteria have been found to predominate in vacuum-packed meat and meat products. In the present study, heterofermentative lactic acid bacteria (subsequently identified with Leuconostoc) were shown to be dominant in beef packed under 80% O_2 : 20% CO_2 and held at -1^o (Fig. 9)

Implicit and extrinsic factors important in spoilage of fresh beef

The above discussions illustrate how the emergence of particular microbial associations on meat can be determined by the storage conditions. Thus it was shown that the flora of fresh beef can be altered from one dominated by Pseudomonas to one consisting mainly of M. thermosphactum. Further, the growth of the last mentioned can in turn be depressed, allowing leuconostocs to become numerically the most important group. The reasons for the prevalence of the above groups stem partly from implicit properties of the organisms and from the interaction of these with the intrinsic and extrinsic factors imposed by the environment. In the following section the

inter-relationships of some of these factors and how these play a major role in determining the composition of the flora is discussed. The comprehensive review by Mossel & Ingram (1955) forms a basis for any consideration of the factors involved in the microbial deterioration of foods and the following draws much from the principles discussed by these authors.

The chemical composition of beef (Table 1) is such that it can be considered to provide a suitable substrate for bacterial growth, and it seems unlikely that nutrient availability or exhaustion would be decisive factors in determining the particular groups of microorganisms which become predominant.

The biochemical changes occurring in post-mortem meat have been the subject of numerous investigations. Although most of these studies are outside the scope of the present work it is useful to discuss briefly some aspects which could have a bearing on the microbiological and organoleptic quality of beef.

With the storage periods and temperatures used in the present study, it seems reasonable to assume that autolytic and/or auto-oxidative changes in beef fat would be of minor significance (Lea, 1931). However, many of the microbial types associated with chilled beef attack fats (Vickery, 1936). This effect can be twofold, firstly a hydrolytic action which gives rise to free fatty acids, and secondly, an oxidative activity which can cause rancidity (Jensen, 1954). These changes are due to the action of lipases and lipoxidases respectively

and they might conceivably contribute to the off-odours associated with spoiled meat.

The fate of proteins in stored meat is uncertain and there is apparently no general agreement as to the extent of autolysis (Davey & Gilbert, 1966). The balance of evidence seems to favour the occurrence of only minor proteolytic changes during the first few weeks post-mortem (Hoagland, McBride & Powick, 1917; Locker, 1960; Jay & Kontou, 1967). Microbial breakdown of native beef proteins is considered to be negligible (Davey & Gilbert, 1966) and any proteolysis which does occur is probably due mainly to the release of cathepsins from lysosomes (De Duve & Beaufay, 1959) as the pH of the muscle falls soon after slaughter. Some controversy also exists as to whether the levels of amino acids change markedly during storage of beef. For example Gardner & Stewart (1966) studied the changes in amino acids in beef held at 4, 9, or 15^{o} and found only small increases in most of the 21 amino acids tested for, the greatest increases being with tryptophan and glutamic acid. The fall in the level of glutamine was noted and thought to be related to production of glutaminase by bacteria. In a later study Jay & Kontou (1967) reported sharp decreases in the amino acid pool in beef stored at 7^{o} for 15 d. These authors followed the changes in 18 amino acids in beef allowed to undergo spoilage. With meat having a low initial bacterial count ($\underline{c}$ 10^{3}/g) there was an increase in the levels of amino acids except cysteine which became undetectable. With high initial numbers of bacteria ($\underline{c}$ 10^{7}/g), all the amino acids except lysine could not be detected by the 15th d. It was concluded that when starting numbers were low a sparing effect on amino acids

at the expense of other compounds (e.g. nucleotides) occurred.

The breakdown *in vivo* of glycogen or "muscle sugar" follows the well documented energy yielding pathways of glycolysis and the Tricarboxylic Acid Cycle; the end products of catabolism being H_2O and CO_2. Shortly after the death of the animal, important physico-chemical changes take place which affect carbohydrate breakdown. Thus when the O_2 supply to the tissues stops, the redox potential falls (Barnes & Ingram, 1955) and aerobic respiration (TCA cycle and oxidative phosphorylation) ceases (Lawrie, 1966). Anaerobic glycolysis continues, lactic acid accumulates and when this reaches sufficient levels a fall in pH (*c* 1.5 units) occurs. The amount of lactic acid produced is related to the glycogen content of the muscle prior to death. Thus if the animal is rested the glycogen content is high and the meat will have a low ultimate pH of around 5.5 - 5.6. On the other hand, if the animal is subjected to stress or exercise, reserves of glycogen will be lower resulting in a higher ultimate pH. It is noteworthy that recent work suggests that the cessation of glycolysis is not due to exhaustion of glycogen reserves, but to a decline in the activities of two enzymes, phosphorylase and phosphofructokinase (Newbold & Scopes, 1967). The importance of pH in all stages of meat technology and microbiology, and many of the phenomena associated with different ultimate pH values are well recognised. These have been reviewed in detail (Bate-Smith, 1948; Ingram, 1962*c*).

The possibility that chemical tests might provide a means for the detection of incipient deterioration has been considered for many years. Several of these techniques are based on estimation of

simple compounds such as H_2S (Weaver, 1927) and NH_3 (Richardson & Scherubel, 1908; Haines, 1931). Other depend on changes in the total (Saffle, May, Hamid & Irby, 1961) or single amino acids such as tryptophan (Gardner & Stewart, 1966). Indirect methods of assessing chemical changes such as determination of extract release volume (Jay, 1966) have also been suggested. In a review of the above and other techniques, Pearson (1968) concluded that determination of free fatty acids, total volatile nitrogen and extract volume might form a basis for assessing meat quality. It is significant however that bacteriological analysis still remains the most widely used method.

Storage temperature is one of the most important extrinsic factors in the preservation of fresh meat. It is well established that as temperature is lowered below optima for microbial growth there is a progressive retardation in rate of multiplication and metabolic activity. Furthermore, such decreases in temperature can have a selective action and, at values of $<5^{o}$ only certain microbial types are capable of growing well. This concept has been appreciated and successfully applied for many years in the preservation of fresh beef. Thus Haines & Smith (1933) investigated the effect of temperature on the time taken for the appearance of slime on lean meat. Small pieces of beef were stored in a saturated atmosphere at temperatures ranging from 0 to 16^{o}. In the range 16 down to 5^{o}, temperature did not greatly affect the time taken for slime formation. Below 5^{o}, a difference of one or two degrees appreciably affected the storage life. For example at 4^{o} slime was evident after 3 - 4 d, but not until 6 - 7 d at 2^{o}. The results could be directly related

to the generation times of the slime-producing organisms, the doubling times at 0 and 20° were 9.1 and 1.3 hours respectively. These observations were later confirmed (Scott, 1937). The growth rates of Achromobacter, Pseudomonas, Candida, Geotrichoides and Mycotorula on ox muscle stored in the range -1 to 30° were compared. Organisms were spray-inoculated onto the surface of the beef to give initial levels of 10^3/sq cm, and the generation times calculated for each organism at -1, 2, 4, 10, 15, 20, 25 and 30°. It was found that the most significant increases in generation times were between 10 and -1°. A detailed investigation of the temperature relationships of the microbial flora and storage life of prepacked beef was conducted by Ayres (1960). Beef steaks were packed in MSAD cellophane or Pliofilm, stored at 0, 5, 10, 15, 20 or 25°, and changes in numbers of microorganisms and the identity of the predominant types studied. With an initial count of c 10^3 bacteria/g, off-odours developed by the 2nd or 3rd d in samples held at 25°. At 0° off-odours were not detected until the 20th d. These odours always occurred before the appearance of slime. The composition of the predominant microflora was also shown to be influenced by the storage temperature. Thus at 15° or above there was approximately equal number of Micrococcus and Pseudomonas. Whereas at the lower temperature (10° or below) the bacteria responsible for slime were almost without exception pseudomonads. In a more recent study (Gardner, 1965) the effects of storage temperature on the aerobic flora of minced beef were assessed. A definite putrid odour was noted when the total bacterial count was of the order of 10^9 organisms/g irrespective of temperature of storage. The time taken to reach this value was found to be c 3 d at 15°. The corresponding time at 4° was c 11 d. Results obtained

in the present work substantiated those discussed above. Furthermore, a knowledge of the generation times of the organisms associated with spoilage can be useful for predicting the approximate shelf-life of beef under a given set of storage conditions.

The growth rates at 4 and -1^{o} of the major microbial groups isolated in the present study are depicted in Fig. 15. These results were obtained by following the increases in numbers in broth cultures (for details of media etc. see Fig. 15) and calculating generation times from:

$$g = \frac{(t_2 - t_1) \log_{10} 2}{\log_{10} n_2/n_1}$$

where g = generation time

n_1 = number of organisms at time t_1

n_2 = number of organisms at time t_2

Temperature has been shown to markedly affect the storage life of moist protein foods other than beef. Thus with studies on poultry it was found that a 12 d extension of shelf-life was obtained with chickens stored at 0^{o} compared with those stored at 10^{o} (Ayres, et al., 1950). Similarly, with fresh fish, the storage temperature has been shown to greatly influence spoilage rates (Shewan, 1961).

Due to their paramount importance in the chilled food industry (Elliot & Michener, 1965) cold tolerant microorganisms have been the subject of many investigations. However, confusion has arisen with the terminology used to describe such organisms, up to the point where it seems that each worker coins his own definition. Microbiological

texts often divide organisms on the basis of the temperature ranges within which growth can occur. Normally three categories are recognised; thermophiles, mesophiles and psychrophiles. Thermophiles are usually clearly distinguishable from mesophiles on optimum growth temperature, which for thermophilic species lies between 55 and 60^{o} (Lamanna & Malette, 1959). The separation of mesophiles and psychrophiles is not as clear cut, since it is difficult to give an exact definition of psychrophilism.

Forster (1887) was apparently the first worker to recognize that some microorganisms were able to grow at low temperature, and he isolated pigmented marine bacteria which multiplied at 0^{o}. Later the term psychrophile was introduced to describe these organisms (Schmidt-Nielson, 1901). The choice of the word psychrophile was perhaps unfortunate (Witter, 1961) since its roots implied that the organisms were "cold-loving" and thus have a low optimum growth temperature. Although microorganisms with optima below 20^{o} have been isolated only rarely, it may well be that investigations of cold habitats and use of appropriate techniques will reveal the widespread existence of obligate psychrophiles (Harder & Veldkamp, 1968). Nevertheless many authors have criticized the use of the word psychrophile (Müller, 1903; Berry & Magoon, 1934; Ingraham & Stokes, 1959; Eddy, 1960; Kandler, 1966) and, although alternatives such as psychrotroph, cold-tolerant and facultative psychrophile have been suggested, none of these has gained general acceptance. In the present study the word psychrophile will be used throughout.

Over the years, investigators have used different criteria to recognize and separate psychrophiles from mesophiles. These are usually based on some evaluation of optimum, minimum or maximum growth temperature, or on changes in growth rate with incubation temperature. Both minimum generation time and maximum cell population have been used to determine optimum temperature. However, as mentioned above, bacteria with optima $< 20^{o}$ have been reported by only a few workers (Sinclair & Stokes, 1964; Hanus & Morita, 1968; Harder & Veldkamp, 1968), and therefore using this definition at present, the incidence of psychrophiles would be unrealistically small. Some authors (e.g. Scott, 1937; Ogilvy & Ayres, 1951; Ingraham, 1958; Camber, 1964) have applied the Arrhenius equation to bacterial growth rates in an attempt to distinguish psychrophiles from mesophiles. Scott (1937) studied the growth of various bacteria and yeasts on ox muscle held at temperatures in the range -1^{o} to 30^{o}. Analysis of the curves of growth rate plotted against temperature suggested that the Arrhenius equation was applicable over a sub-optimal temperature range. These results were later confirmed (Ogilvy & Ayres, 1951) with the growth of slime forming bacteria on refrigerated chicken. Ingraham (1958) believed that the slope (or temperature co-efficient, μ) of an Arrhenius plot might be constant for any particular organism. Working with a mesophilic Escherichia coli and a psychrophilic Ps.fluorescens, he claimed that the temperature co-efficient was significantly higher with the mesophile. In other words the effect of decreasing temperature on growth rate was more marked with E. coli. These findings have since been contested (Hanus & Morita, 1968). The growth characteristics of strains of vibrio (an obligate psychrophile,

a facultative psychrophile and a mesophile) were studied. From the results, it was concluded that the μ values did not reflect whether or not an organism was psychrophilic.

From a practical point of view it would seem the best definition of a psychrophile would be one stemming simply from the ability of an organism to grow at low temperature. Thus Ingraham & Stokes (1959) proposed that a psychrophilic microorganism is one which grows reasonably well at 0^{o} or has a generation time of $<$ 48 h at this temperature. It has been concluded that this is a property possessed by a rather limited group of bacteria, mainly non-sporing Gram negative rods in particular members of the genus Pseudomonas (Ingraham & Stokes, 1959; Rose, 1968). However, evidence is accumulating which suggests that psychrophilism is ubiquitous in nature and includes representatives of a wide range of bacterial genera and species (Stokes & Redmond, 1966).

Investigations have and are being made into the biochemical basis of low temperature growth (Farrell & Rose, 1965), but to date these studies have not yet fully explained the property of psychrophilism.

Although low temperature is currently the principal method for preserving beef, the shelf-life achieved is still limited. There is therefore a need for novel and better preservation techniques. In this context, modification of the atmosphere by gas-packing would seem to be promising.

As stressed by Ingram (1962a) the data derived from many

investigations of the microbiology of prepacked meat are of little comparative value since the majority of workers did not monitor changes in the internal atmosphere of the pack. In addition to influencing the rate of microbial growth, the gaseous environment can markedly affect the composition of the flora as well as the appearance of fresh beef. The components of particular interest are CO_2 and O_2 and, during storage of prepacked beef, changes in their concentrations are governed by a complex interrelationship of bacterial respiration, gaseous exchange by the tissues and the permeability of the wrapping film. The absorption of O_2 by meat is attributed to combined effects of microbial respiration and residual oxidative enzymes in the meat. Recently the role of meat enzymes was discounted (Gardner & Carson, 1967). These authors concluded that enzyme activity was not detectable in "microbe free" porcine muscle stored at 2^{o}. If their data are examined it can be deduced that O_2 uptake by the muscle had occurred. After 3 days storage the O_2 had fallen to 19.2% (Dr. G.A. Gardner, pers. comm.). This represents a consumption of c 0.2 ml which in the experiments of Gardner and Carson is equivalent to c 1.0 µl/h/g of tissue. It is noteworthy that in earlier studies (Urbin & Wilson, 1958) the post-mortem requirements of bovine tissue held at 1^{o} were reported to be 0.7 µl/h/g of wet tissue, a figure which is of the same order as that given above. Of the oxidative enzymes surviving in post-mortem muscle, the data of Grant (1955) indicates that succinic dehydrogenase was the most active.

Concomitant with O_2 uptake, freshly cut meat evolves appreciable amounts of CO_2. The rate of CO_2 production has been reported to be 1.1 µl/h/g of tissue at 1^{o} (Urbin & Wilson, 1958) and was considered

to be derived mainly from the dissociation of bicarbonate occurring as the tissues became more acid due to lactate accumulation.

Microorganisms present on meat may also consume O_2 and produce CO_2. If contamination is low then gas changes as a result of microbial activity will be comparatively unimportant. When populations are of the order of 10^7/g (bacon) it has been calculated (Ingram, 1962a) that the rate of O_2 consumption attributable to this population is 0.15 ml/g of bacon (stored 4 d at 15°). In the present investigations (Fig. 7) with prepacked beef stored for 12 d at 4°, O_2 was consumed at a rate of approximately 0.2 ml/g of beef (c/f 0.15 ml/g quoted by Ingram). The rate of O_2 consumption is dependent not only on numbers but also on the types of microorganisms present. For example, it can be established that the O_2 uptake by a pseudomonad cell is 6 times that of a yeast cell. Thus Brock (1966) quoted QO_2 values for Bacillus fluorescens non liquefaciens (Pseudomonas putida) and Saccharomyces cerevisiae as being 4100 ml and 10 ml O_2/h/g wet weight of cells respectively. The wet weight of a pseuodomonad cell is c 1 x 10^{-12}g, and that of the yeast 70 x 10^{-12}g. Thus the ratio of O_2 consumed by a pseudomonad cell to a yeast cell is given by

$$\frac{1 \times 10^{-12} \times 4100}{70 \times 10^{12} \times 10}$$

which approximates to 6. CO_2 production in prepacked meats can also be partly due to microbial activity. The build-up of CO_2 is particularly notable when meat is enclosed in a gas impermeable film (Fig. 4). However even with highly gas permeable films an increase in CO_2 concentration was noted (Fig. 4), and it can therefore be deduced that the rate of CO_2 diffusion through the permeable film was

less than CO_2 production by the meat and its associated microflora.

The pattern of gaseous exchange in prepacked beef is similar in many ways to that occurring in prepacked pork (Gardner et al., 1967). With samples wrapped in impermeable film and stored at 2° for 8 d, the O_2 tension fell to < 5% and CO_2 increased to c 12%. The latter also increased when a permeable film was used, reaching values of c 15% after 4–6 d. However, in these packs the O_2 decreased by only a few percent. This finding is in contrast to those reported here, where it can be seen (Fig. 4,6,7) that with beef packed with Visqueen Meatwrap (permeable film) the O_2 fell from an initial value of 20% to c 1%.

As was mentioned previously, changes in internal gas composition can have marked effects on both the numbers and types of microorganisms present. It is believed that the inhibition of the aerobic spoilage bacteria is due to the high CO_2 concentration rather than the low O_2 levels (Ingram, 1962; Gardner & Carson, 1967; Shaw & Nicol, 1969). The inhibitory effects of CO_2 have been known for many years and the early work (Valley & Rettger, 1927; Killefer, 1930; Coyne, 1933; Haines, 1933b; Empey & Vickery, 1933) led to the commercial application of CO_2 as a food preservative. Its use in retarding spoilage of prepacked meat was investigated by Ogilvy & Ayres (1950). These authors assessed the effectiveness of different concentrations of CO_2(5, 15, 25%) in prolonging the shelf-life of cut-up chicken and found that storage index (ratio of keeping time is CO_2 to that in air) was a linear function of CO_2 concentration. In addition the results suggested that the inhibitory effect of CO_2 was enhanced by a

reduction in the storage temperature.

The inhibitory action of CO_2 is not fully understood (Wimpenny, 1969). Early interpretations (e.g. Valley & Rettger, 1927) that its effects were due to reduction in pH have since been questioned (King & Nagel, 1967). Other explanations can be offered. For example, in any metabolic reaction yielding CO_2 it could be considered that the presence of a high CO_2 concentration would alter the equilibrium (by the Law of Mass Action) thus inhibiting to some degree at least these reactions. In addition, CO_2 might have a specific inhibitory effect on certain enzymes (e.g. permeases)or perhaps act as an end-product repressor. Any one of these could result in death or retardation of growth of susceptible microorganisms.

Although carbon dioxide appreciably delays the time taken for meat to spoil, there are disadvantages related to the use of this gas for preserving red meat. Thus, atmospheres containing $> 25\%$ CO_2 can accelerate the formation of brown metmyoglobin (Moran, 1935, 1938)A similar situation may arise when gas impermeable film is used to pack beef, especially where the film is in contact with the meat. To maintain an acceptable red colour it is therefore necessary to delay the formation of metmyoglobin. This can be achieved by anaerobic or vacuum packing. Under these conditions the pigment is held in the myoglobin state and when the pack is opened and exposed to air, oxymyoglobin is regenerated (Rikert, Ball & Stier, 1957; Pirko & Ayres, 1957; Cutaia & Ordal, 1964). However, it appears that the purplish colour of such beef is not generally accepted by the consumer. Other gas storage techniques for preservation of colour have been

reported. For example, the use of CO containing air has been suggested (El-Badawi, Cain, Samuels & Anglemeier 1964). These studies showed this gas mixture could stabilize beef colour for up to 15 d. However, as the authors pointed out, in using CO to preserve beef, consideration must be given to possible public health hazards, thus toxicity testing would be essential. Recent work in Australia (Kaess & Weidemann, 1968) re-examined the possibility of using low concentrations of $O_3 \pm CO_2$ to delay microbial deterioration and surface discolouration of chilled beef. The results showed that the beneficial effects of O_3 were at best marginal. As the O_3 concentration was increased the time taken for the onset of discolouration shortened, thus limiting the concentrations which could be used. At those levels, the retarding effect of the gas on the growth of the main spoilage organisms (non-pigmented Pseudomonas) was similar to that obtained using 11% CO_2.

In the work reported here the use of gas mixture with high O_2 content and sufficient CO_2 to inhibit growth of the aerobic spoilage bacteria has been assessed. Mixtures with high initial O_2 tension were chosen since it was reasoned that this might overcome O_2 depletion by microorganisms and help to maintain the pigment in the oxygenated myoglobin state. It is worth noting that discolouration of beef (i.e. disappearance of oxymyoglobin and/or formation of metmyoglobin) is unlikely to be caused by bacterial metabolism of the pigment. Collaborative work done elsewhere in this laboratory lends support to this hypothesis. In these experiments a pseudomonad typical of those isolated from beef was grown in a nutrient broth containing oxymyoglobin. It was shown that oxymyoglobin

disappearance did not occur until the O_2 level had fallen to $< 5\%$, even though the pseudomonad had grown normally (i.e. a 10^3 increase in numbers). These results confirm an earlier report by Robach & Costilow (1961) who concluded that pigment change in fresh beef was due to reduction in O_2 tension by microbial growth and/or by physical means.

From a consideration of the work reported here and the literature discussed above the following general conclusions may be drawn. Firstly, the deterioration of chilled beef, in particular off-odour production, discolouration and sliminess, is associated with the growth and activities of microorganisms. Secondly, the interaction of various intrinsic and extrinsic factors can markedly affect the composition and multiplication rate of the microbial population. Finally, appreciable extensions in storage life of fresh beef can be achieved by manipulation of the environment e.g. low temperature storage and packing in certain gas mixtures.

TABLE 1

CHEMICAL COMPOSITION OF MAMMALIAN MUSCLE AFTER RIGOR MORTIS *

		%
Water		75.5
Protein		18.0
Fat		3.0
Soluble non-protein substances		3.5
– nitrogenous	1.6%	
– carbohydrate	1.2%	
– inorganic	0.6%	
– Traces of glycolitic intermediates, trace metals, vitamins etc.	0.1%	

* Source, Lawrie (1966)

Fig. 1. Changes in numbers of microorganisms on beef wrapped in permeable film. Each point is the result obtained from one sample and the spread of results at each sampling time is shown by the hatching. Closed triangle, low initial infection; closed circles, medium to high initial infection, and crosses, samples judged unacceptable.

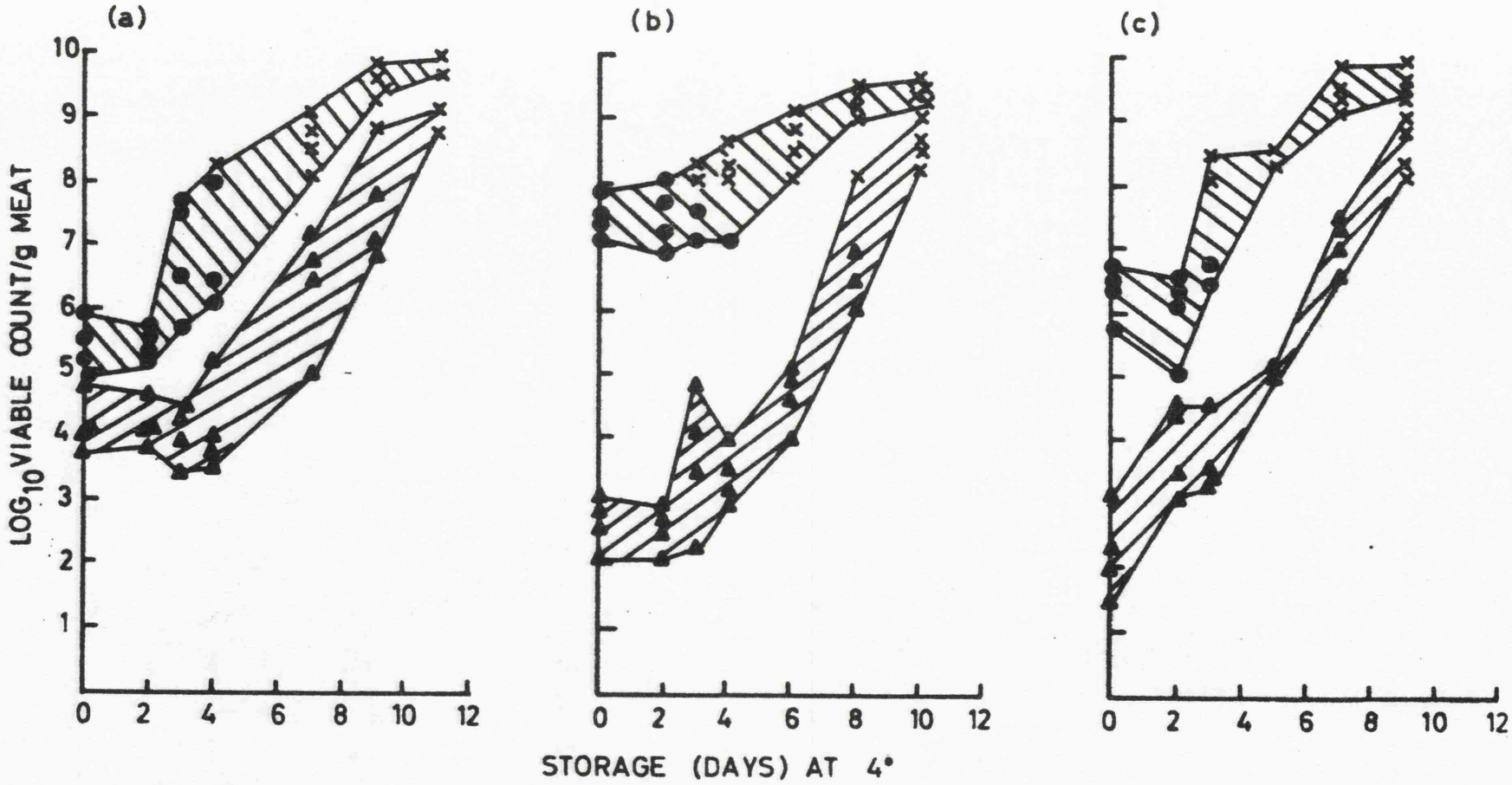


Fig.1. CHANGES IN NUMBERS OF MICROORGANISMS ON BEEF WRAPPED IN PERMEABLE FILM.

Fig. 2. Influence of temperature on microbial growth on meat. Each point is the result obtained from one sample and the spread of results at each sampling time is shown by the hatching. Samples judged acceptable (closed circles) and unacceptable (crosses).

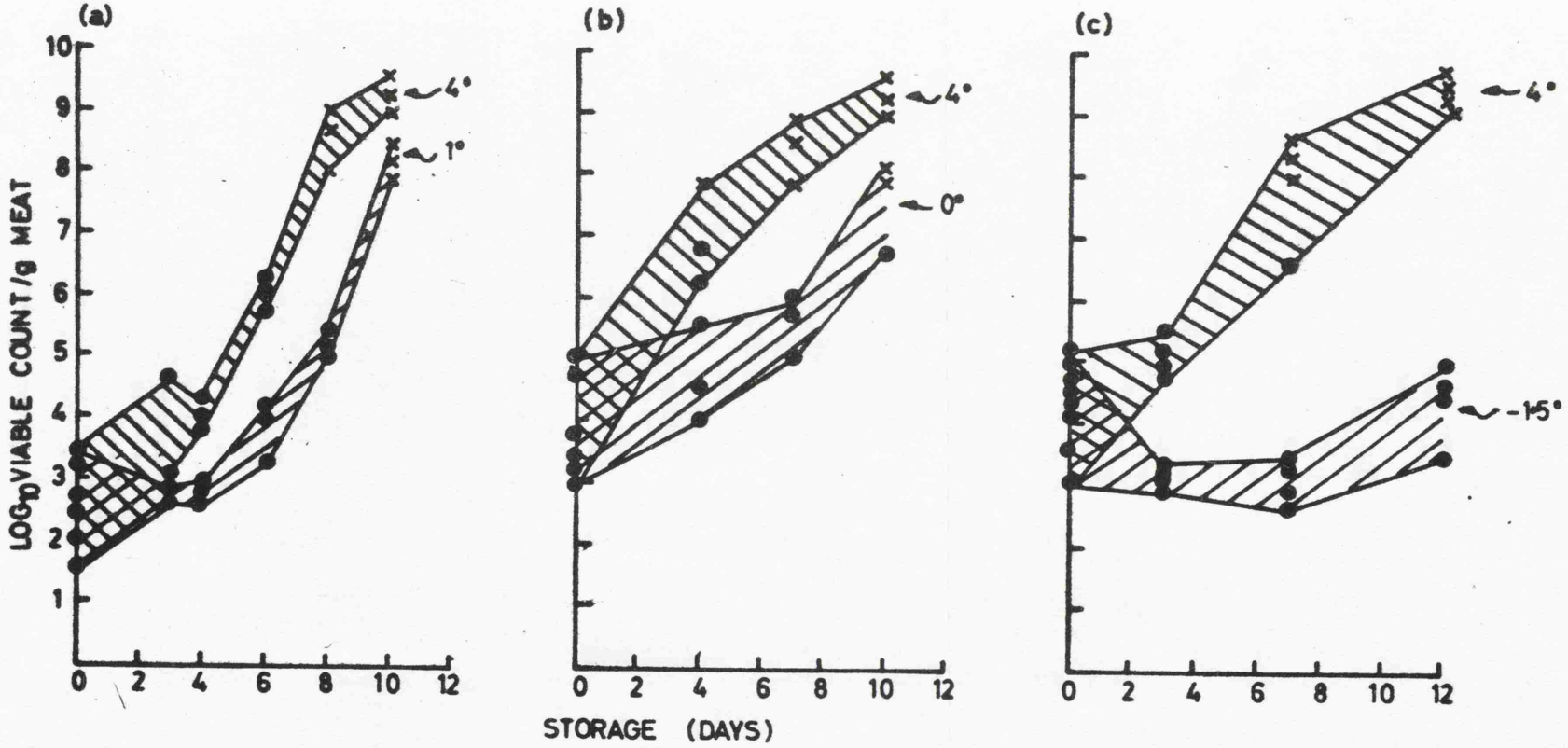


Fig.2. INFLUENCE OF TEMPERATURE ON MICROBIAL GROWTH IN MEAT.

Fig. 3. Influence of film permeability on characteristics of stored meat. Each point represents mean of 4 samples.

(a) Open circles, O_2, gas permeable film; closed circles, CO_2, gas permeable film; open squares, O_2, gas impermeable film; closed squares, CO_2, gas impermeable film.

(b) Open triangles, viable count, meat wrapped with permeable film; closed triangles, viable count, meat wrapped with impermeable film. Number of samples judged unacceptable shown in squares.

(c) Open, Gram negative; shaded, Gram positive; I, permeable packs; II, impermeable packs.

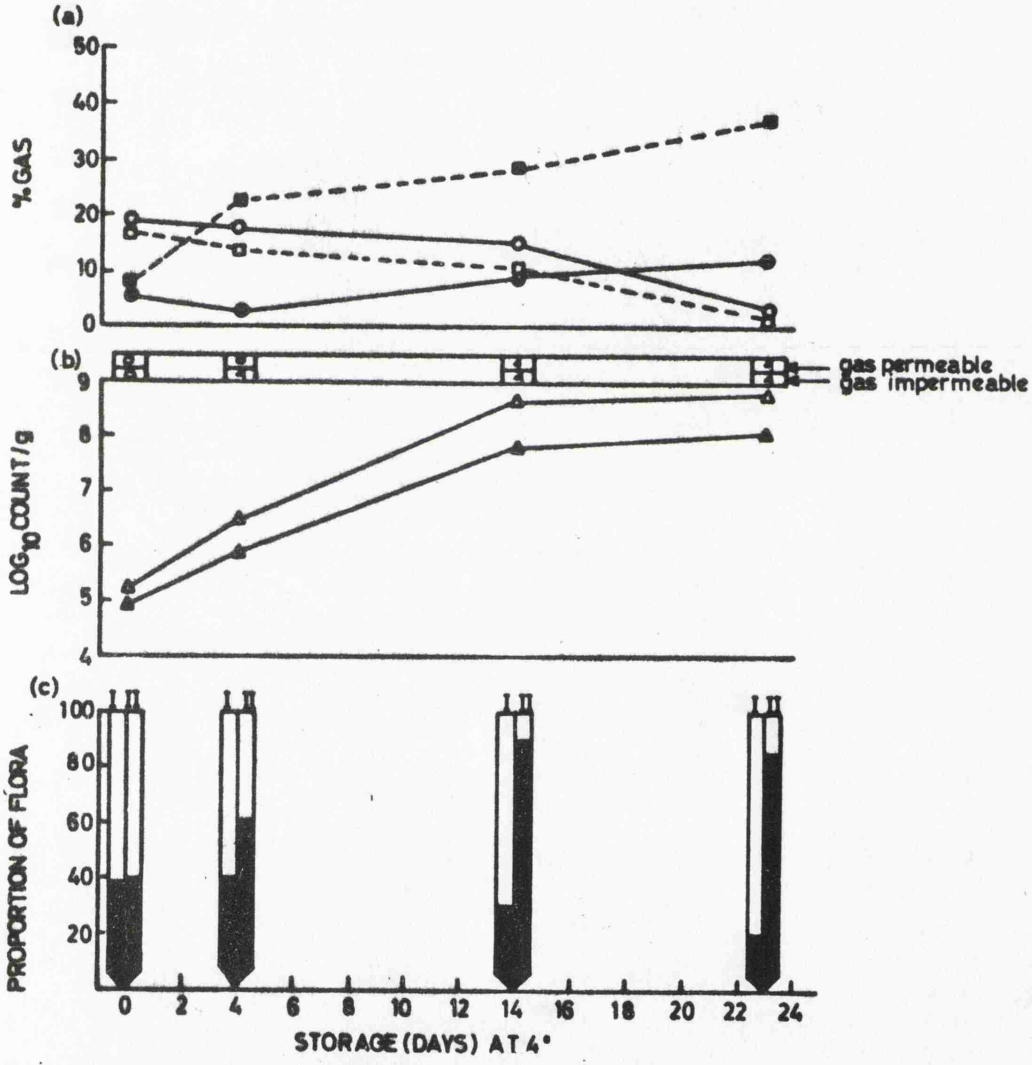


Fig.3. INFLUENCE OF FILM PERMEABILITY ON CHARACTERISTICS OF STORED MEAT.

Fig. 4. Influence of film permeability on characteristics of stored meat. Each point in the graphs represents mean of 4 samples.

(a) Open circles, O_2, gas permeable film; closed circles, CO_2 gas permeable film; open squares, O_2, gas impermeable film; closed squares, CO_2 gas impermeable film.

(b) Open triangles, viable count, meat wrapped with permeable film; closed triangles, viable count, meat wrapped with impermeable film. Number of samples judged unacceptable shown in squares.

(c) Diagonal hatching, Pseudomonas; shaded, Microbacterium thermosphactum; vertical hatching, Micrococcus; dots, Enterobacteriaceae; open, Acinetobacter; I, permeable packs; II, impermeable packs. 20 isolates per sample. Total of 640 isolates identified.

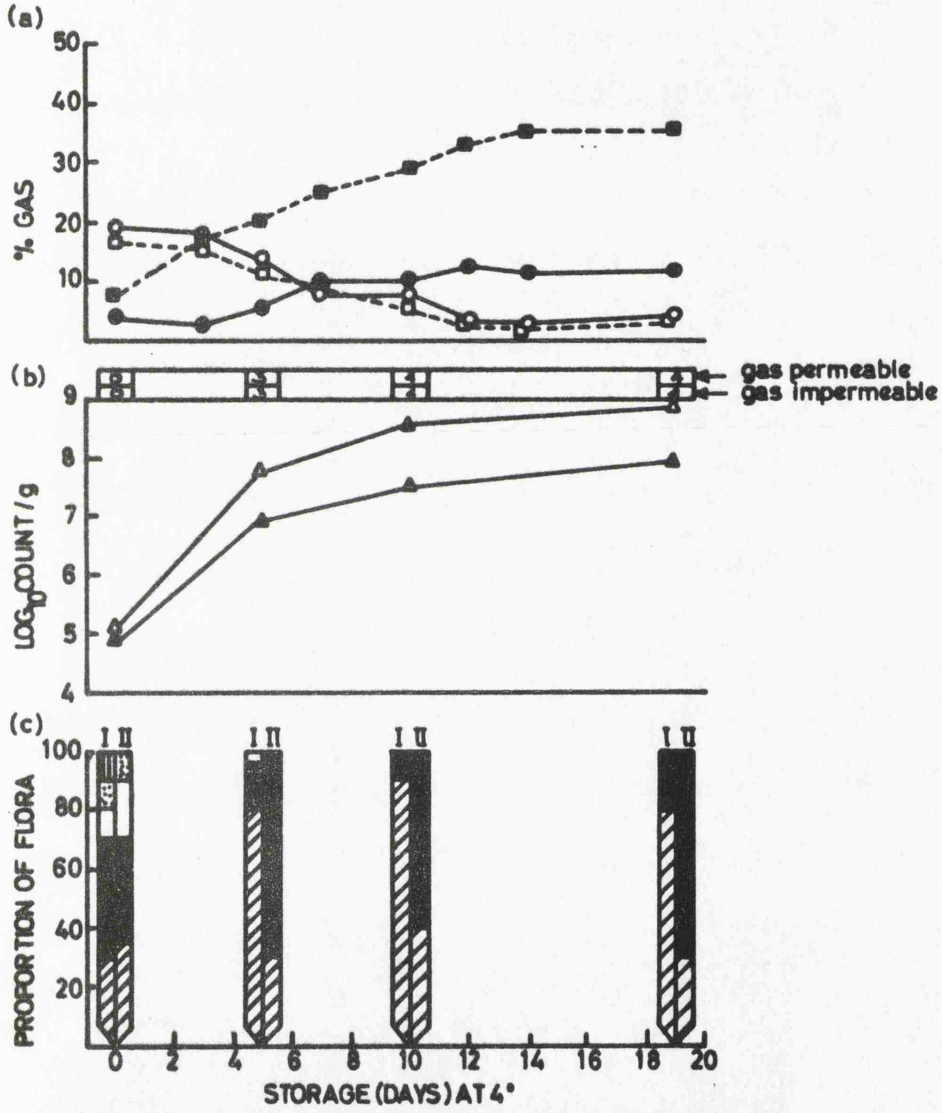


Fig.4. INFLUENCE OF FILM PERMEABILITY ON CHARACTERISTICS OF STORED MEAT.

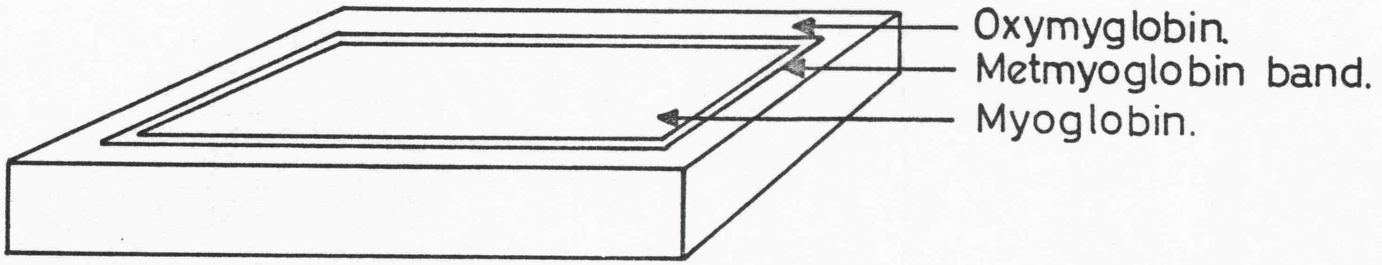


Fig.5 DIAGRAMMATIC REPRESENTATION OF PIGMENTS IN BEEF PACKED WITH GAS IMPERMEABLE FILM.

Fig. 6. Effects of gas packing in 60% N_2 + 20% 0_2+ 20% CO_2. Each point represents mean of 4 samples.

(a) Open circles, 0_2, air packed; closed circles, CO_2, air packed. Open squares, 0_2, gas packed; closed squares, CO_2, gas packed.

(b) Open triangles, viable count, air packed; closed triangles, viable count, gas packed. Number of samples judged unacceptable shown in squares.

(c) Diagonal hatching, Pseudomonas; shaded, Microbacterium thermosphactum; open, Acinetobacter; vertical hatching, Micrococcus; I,air packed; II,gas packed. 20 isolates per sample, total of 480 isolates identified.

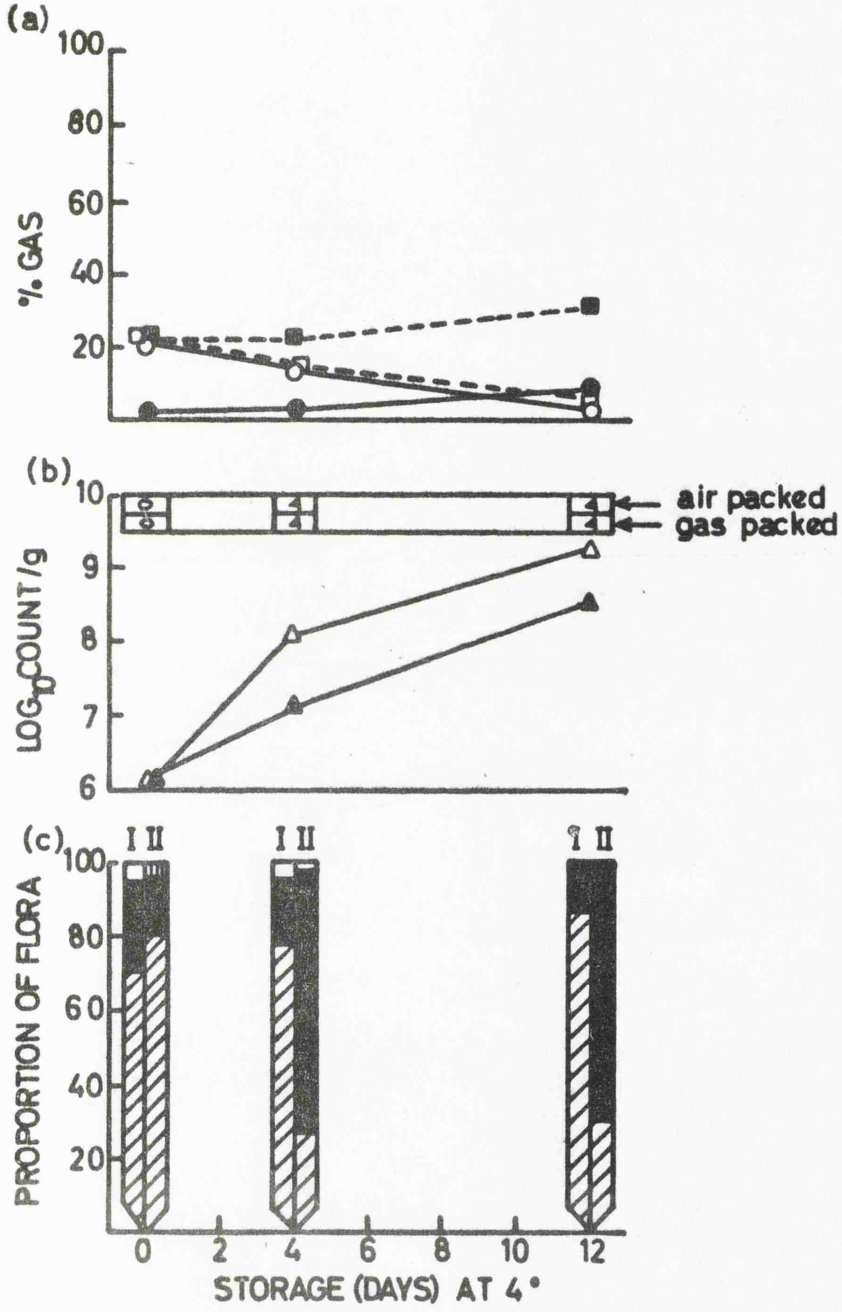


Fig.6. EFFECTS OF GAS PACKING IN 60% N_2 + 20% O_2 + 20% CO_2

Fig. 7. Effects of gas packing in 80% O_2 + 20% CO_2.

Each point represents mean of 4 samples.

(a) Open circles, O_2, air packed; closed circles, CO_2, air packed. Open squares, O_2, gas packed; closed squares, CO_2, gas packed.

(b) Open triangles, viable count, air packed; closed triangles, viable count, gas packed. Number of samples judged unacceptable shown in squares.

(c) Diagonal hatching, Pseudomonas; shaded, Microbacterium thermosphactum; open, Acinetobacter; vertical hatching, Micrococcus; dots, Enterobacteriaceae; I, air packed; II,gas packed. 20 isolates per sample, total of 960 isolates identified.

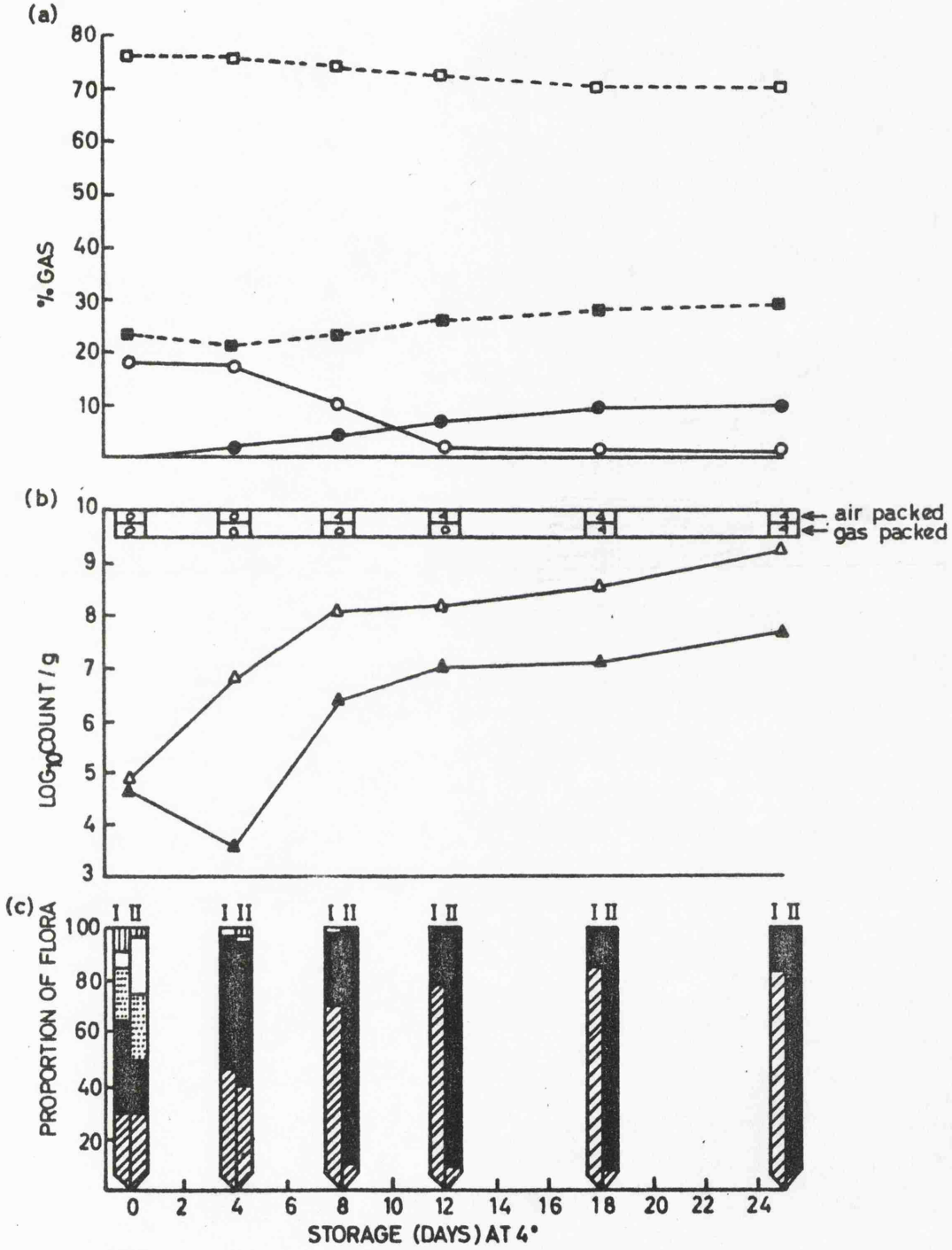


Fig.7. EFFECTS OF GAS PACKING IN 80% O_2 + 20% CO_2

Fig. 8. Effects of gas packing in 80% O_2 + 20% N_2.

Each point represents mean of 4 samples.

(a) Open circles, O_2, air packed; closed circles, CO_2, air packed. Open squares, O_2, gas packed; closed squares, CO_2, gas packed.

(b) Open triangles, viable count, air packed; closed triangles, viable count, gas packed. Number of samples judged unacceptable shown in squares.

(c) Diagonal hatching, Pseudomonas; shaded, Microbacterium thermosphactum; dots, Enterobacteriaceae; open, Acinetobacter, I, air packed; II, gas packed. 20 isolates per sample, total of 640 isolates identified.

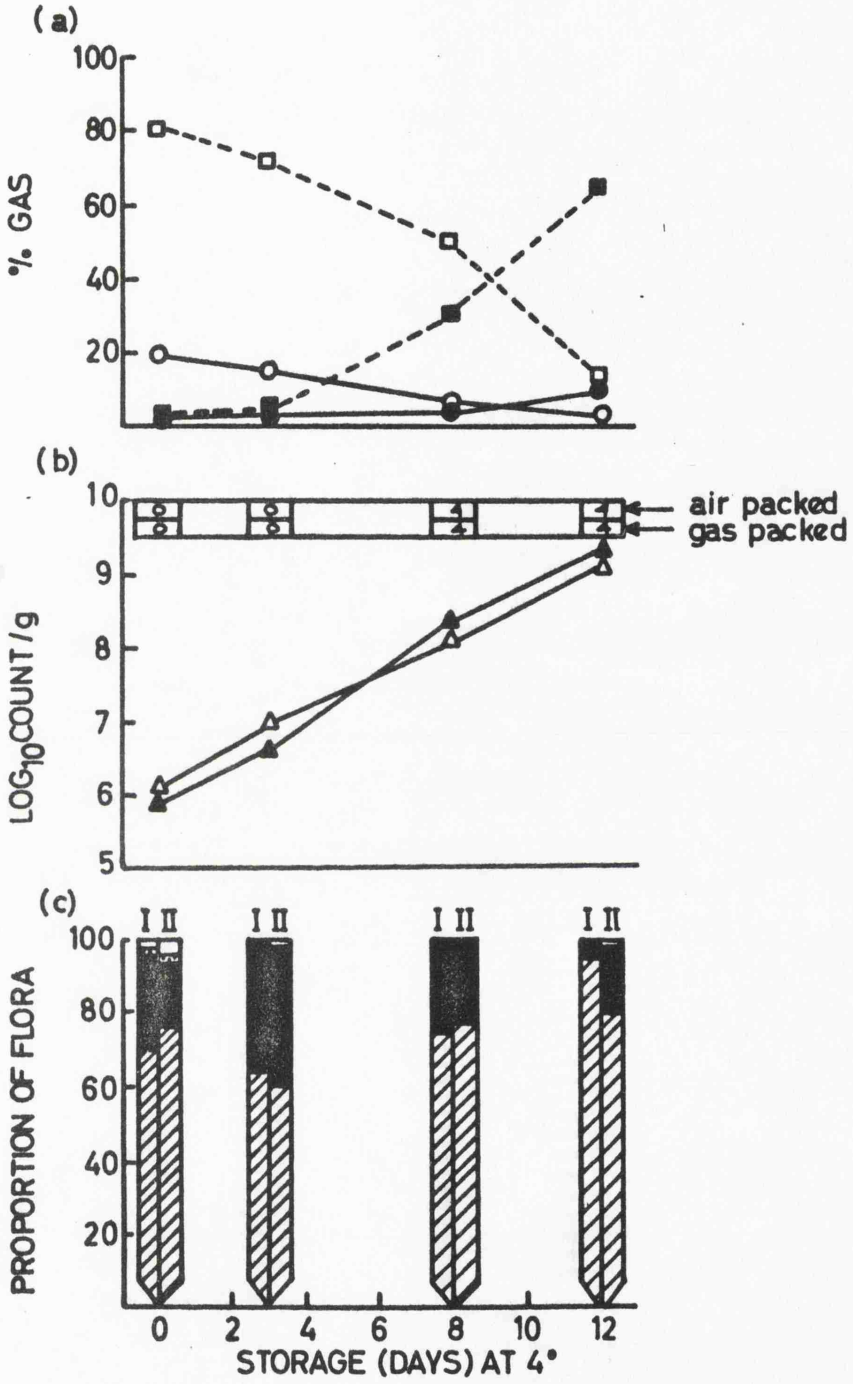


Fig.8. EFFECTS OF GAS PACKING IN 80% O_2 + 20% N_2

Fig. 9. Effects of gas packing in 80% O_2 + 20% CO_2 and storage temperature.

(a_1), (a_2) Open circles, O_2, air packed; closed circles, CO_2, air packed. Open squares, O_2, gas packed; closed squares, CO_2, gas packed.

(b_1), (b_2) Open triangles, viable count, air packed; closed triangles, viable count, gas packed. Number of samples judged unacceptable shown in squares.

(c_1), (c_2) Diagonal hatching, Pseudomonas; shaded, Microbacterium thermosphactum; open, Acinetobacter; horizontal hatching, Leuconostoc; I, air packed; II, gas packed. 20 isolates per sample, total of 2,240 isolates identified.

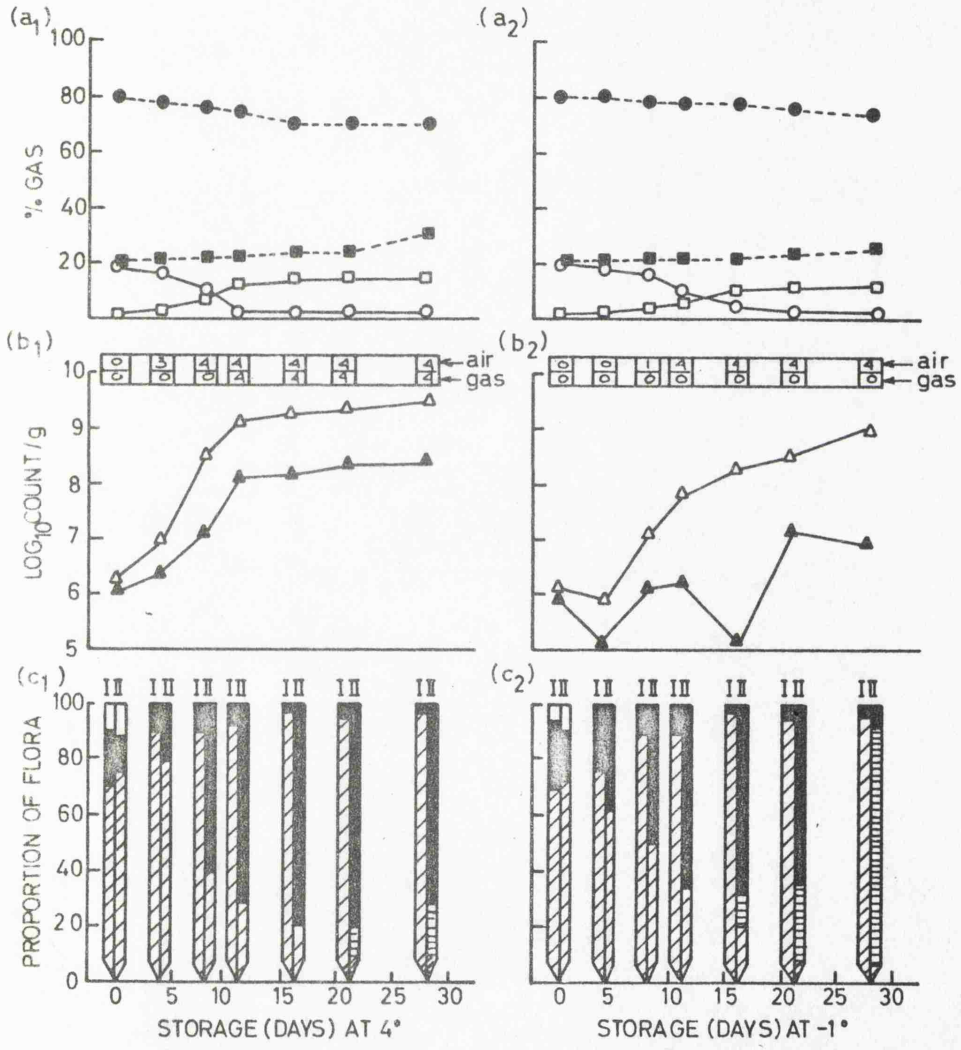
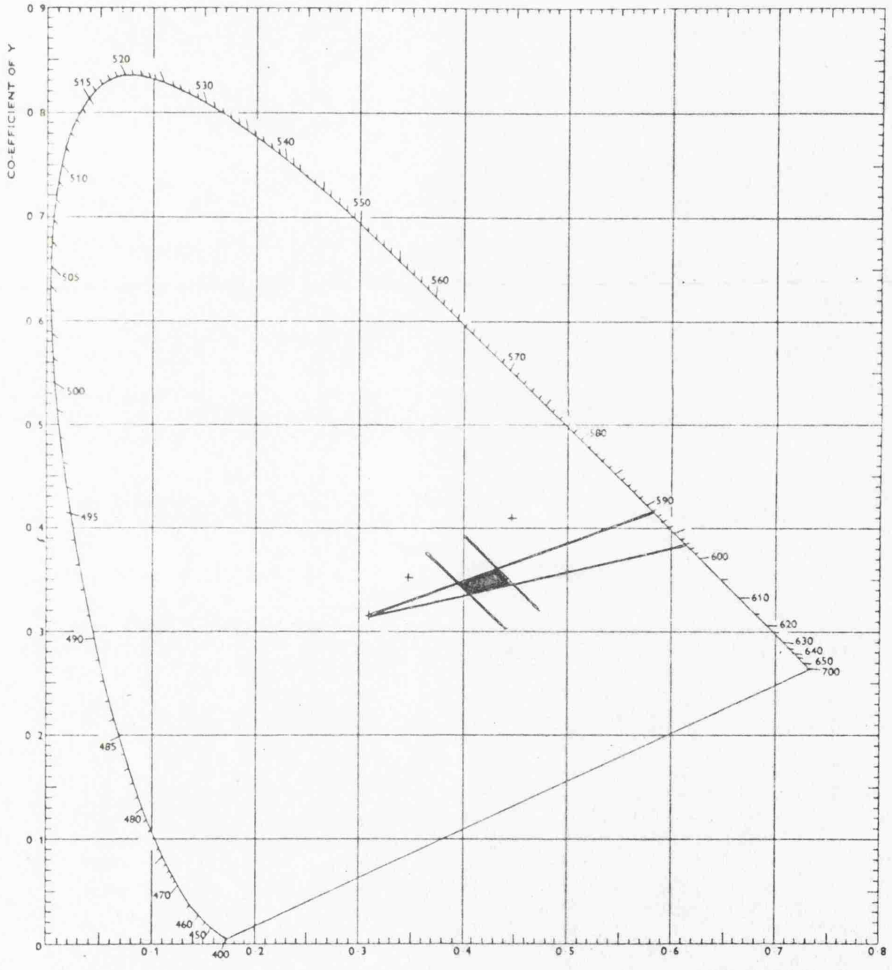


Fig.9. EFFECTS OF GAS PACKING IN 80% + 20% CO_2 AND STORAGE TEMPERATURE.

COLORCORD

CHROMATICITY DIAGRAM

DATE:—			
SAMPLE:—			
INSTRUMENT READING			
X			
Y			
Z			



DATA SHEET NO. 63. C.I.E. TRICHROMATIC COORDINATES WITH SPECTRUM WAVELENGTHS IN MILLIMICRONS AND STANDARD ILLUMINANTS A, B AND C

Fig.10. DIAGRAM SHOWING WHERE BEEF CHROMATICITIES LIE (shaded area)

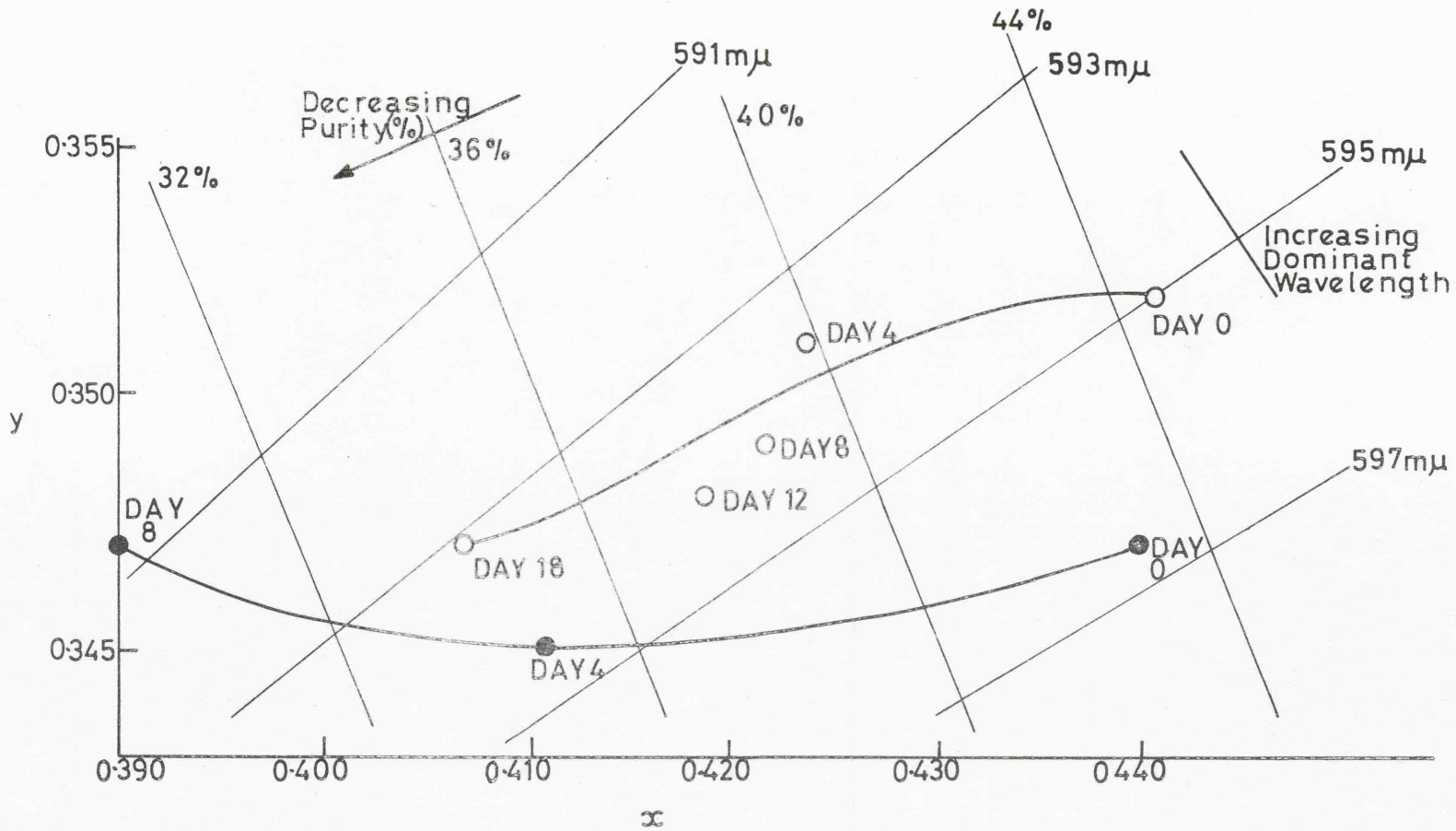


Fig.11. EXPANDED CHROMATICITY DIAGRAM (Open circles, gas packed. Closed circles, air packed.)

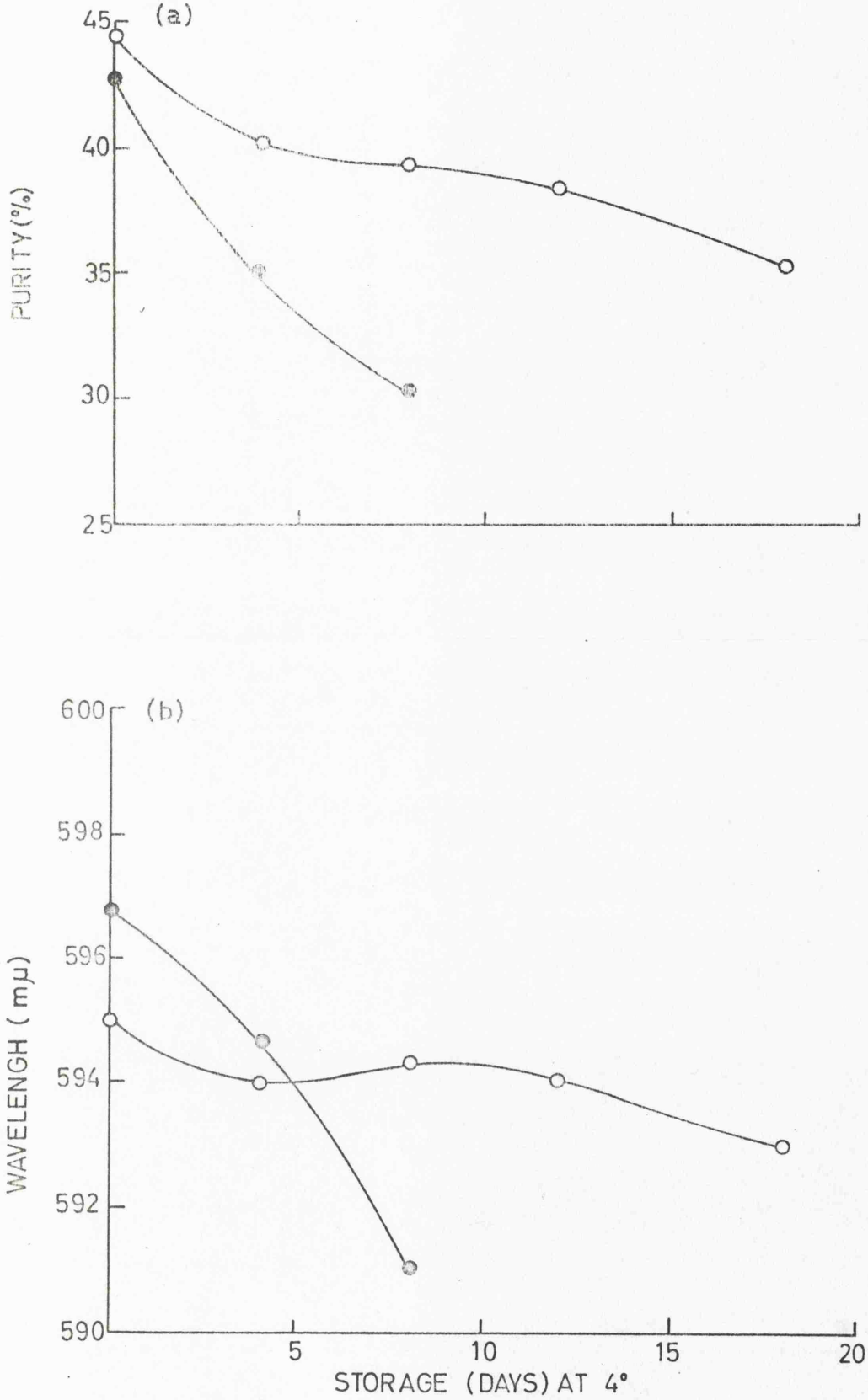


Fig.12. CHANGES IN DOMINANT WAVELENGH AND PURITY OF GAS PACKED (80% O_2 + 20% CO_2) AND AIR PACKED BEEF. (Open circles, gas packed; closed circles, air packed.)

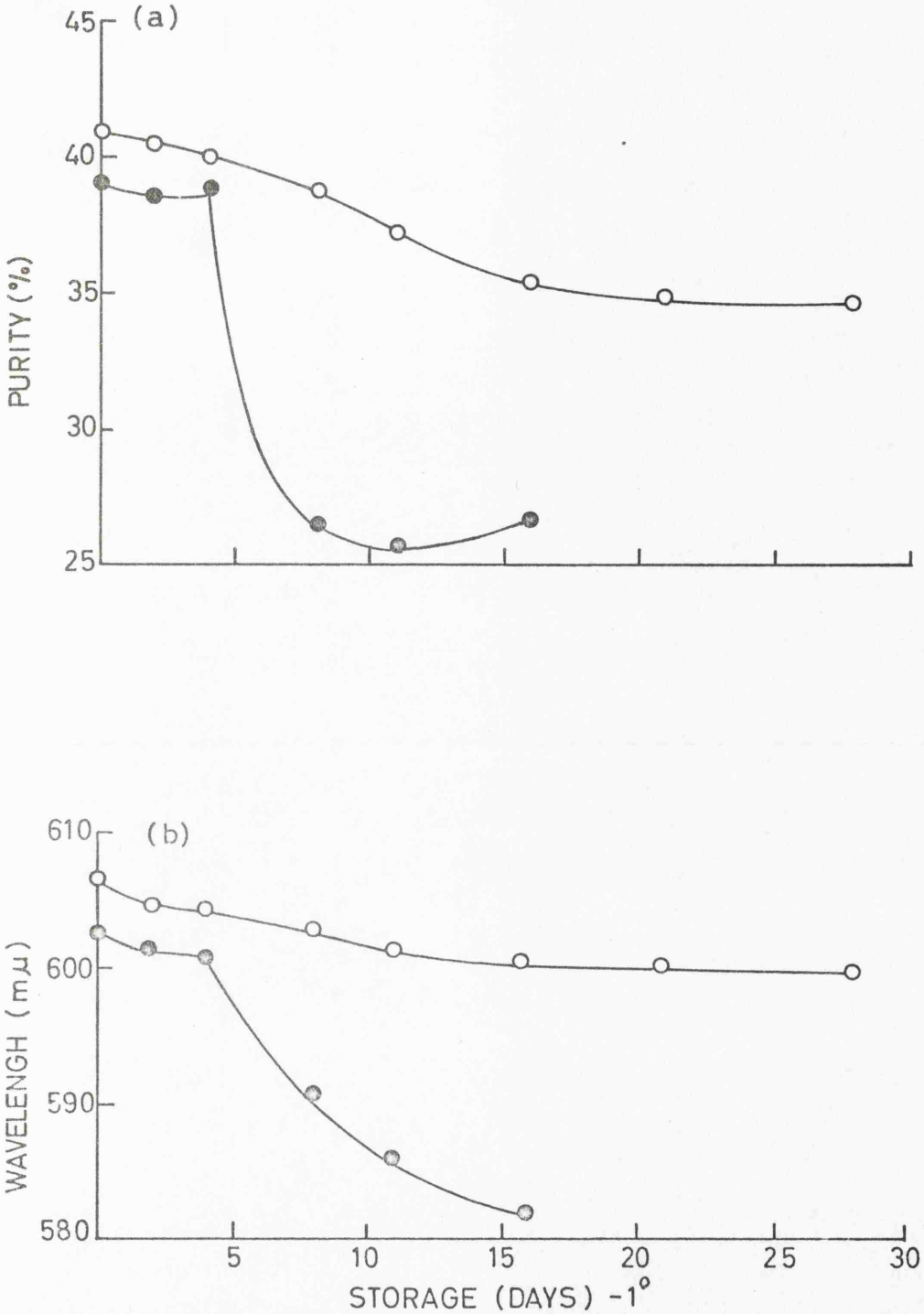


Fig.13. CHANGES IN DOMINANT WAVELENGH AND PURITY OF GAS PACKED (open circles) AND AIR PACKED (closed circles) BEEF

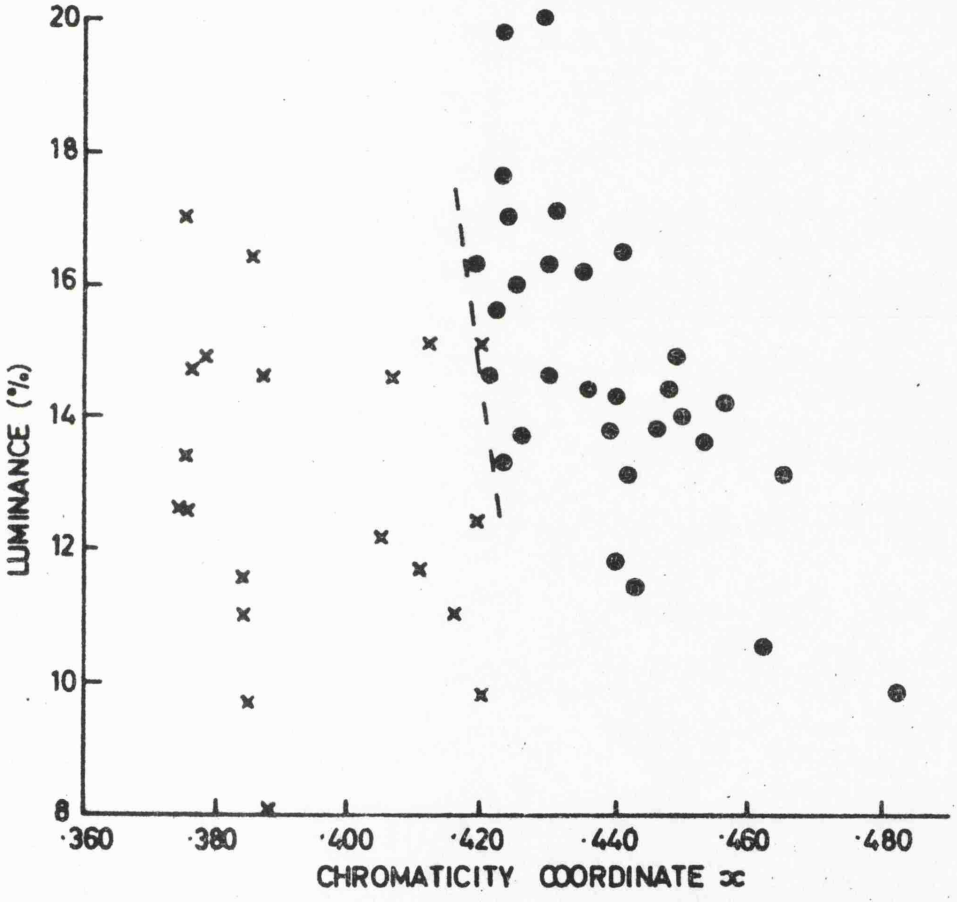


Fig.14. COLOUR OF FRESH BEEF. DISCRIMINATION DIAGRAM OF LUMINANCE AND CHROMATICITY COORDINATE x (Dots, acceptable samples, crosses unacceptable samples.)

Fig. 15. Growth rates of representative bacteria isolated from meat. Cultures were grown with shaking in a thermostatically controlled water/glycerol bath. Pseudomonas and Acinetobacter were grown in Heart Infusion Broth (100 ml in 250 ml conical flasks). Increase in numbers was determined by surface plate counts on Heart Infusion Agar. Leuconostoc and M. thermosphactum were grown in APT broth and counted on APT agar. Generation times for each organism were calculated and the time taken for a 10^5 increase in population determined.

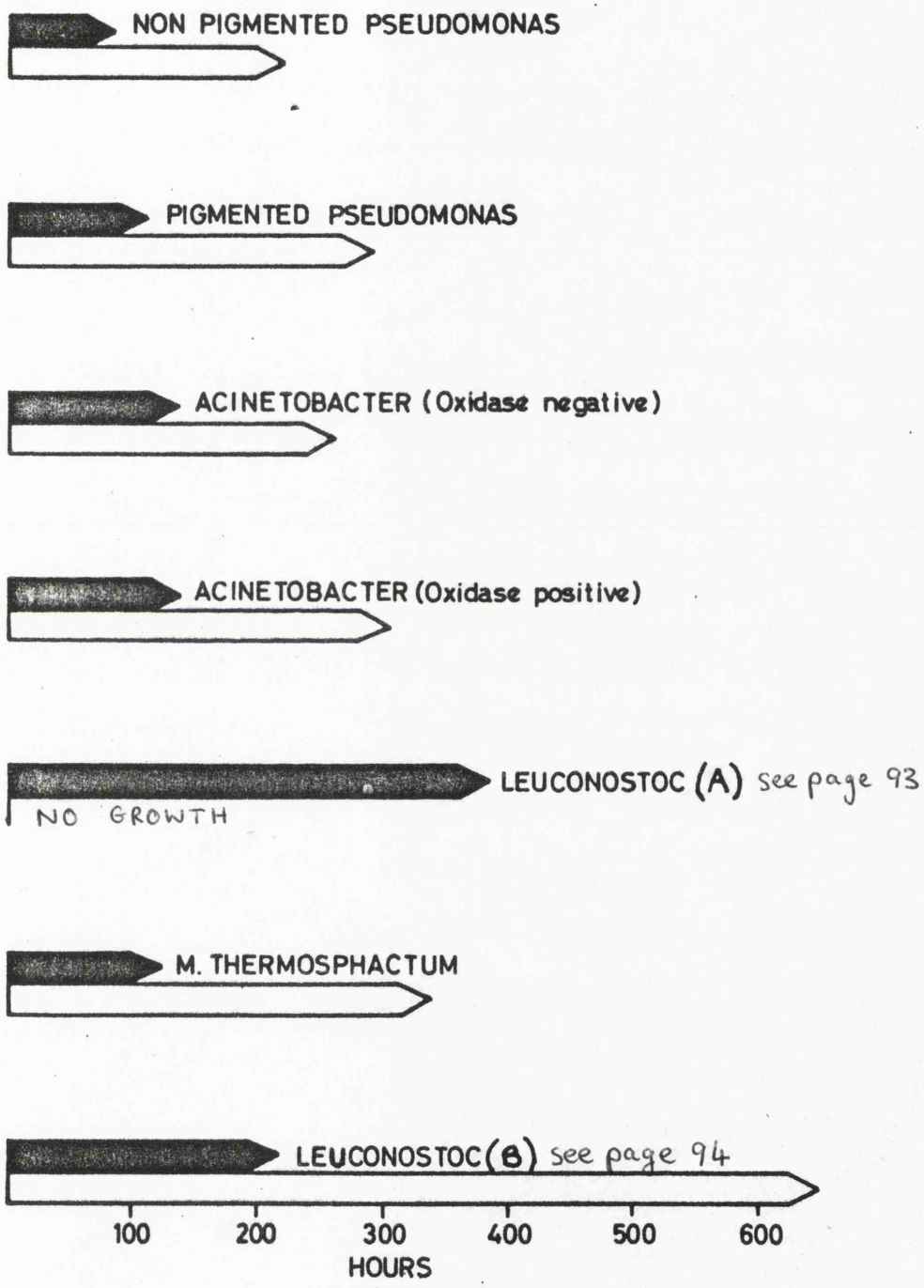


Fig. 15. GROWTH RATES OF REPRESENTATIVE BACTERIA ISOLATED FROM MEAT. ARROWS INDICATE THE TIME TAKEN FOR EACH TYPE TO REACH A POPULATION OF 10^8 FROM INITIAL NUMBER OF 10^3. SHADED, AT 4°; OPEN, AT -1°

S E C T I O N B

PROPERTIES AND IDENTIFICATION OF MICROORGANISMS RECOVERED FROM BEEF

INTRODUCTION

The following section deals with the detailed morphological and physiological properties of representative strains of microorganisms which became dominant under a given set of storage conditions. Less emphasis was placed on those bacteria e.g. Micrococcus and Enterobacteriaceae which although often isolated, did not form significant proportions of the microbial association of spoiled beef. Organisms which were recovered only occasionally such as Aeromonas, Staphylococcus and Bacillus were not studied beyond preliminary identification stage.

METHODS AND MATERIALS

The following tests were applied to all the isolates.

Gram Stain. Jensen's modification (Cruickshank, 1965) was used.

Motility and morphology. Suspensions of organisms present in the moisture collecting at the base of Heart Infusion Agar (Difco) slope cultures were examined with a phase contrast microscope.

Catalase. Hydrogen peroxide (10% v/v) was added to colonies on Heart Infusion or APT (Evans & Niven, 1951) agar and the evolution of gas was taken as evidence of the action of catalase.

ENTEROBACTERIACEAE

The inoculum for all tests was taken from 18 h Heart Infusion Broth cultures, and 30^{o} was the incubation temperature throughout.

Flagella. Organisms grown on HIA slopes for 18–24 h were harvested in 0.5% (v/v) formalin, and the suspension diluted to a suitable strength. A drop of the suspension was placed at the end of a clean slide which was inclined so that the descent of the drop left a thin film. This was air-dried and then fixed with Kirkpatrick's fixative (60 ml ethanol, 30 ml chloroform, 10 ml formalin) for 5 minutes. The fixative was washed off with distilled water. One part Plimmer's mordant (Plimmer & Paine, 1921) was thoroughly mixed with 3 parts distilled water in a conical flask and agitated for 1 minute. The 1:3 ratio was recommended by Mr. W. Hodgkiss (pers. comm.). The diluted mordant was filtered on to the slide and, after 5 min, the films were washed, stained with Ziehls carbol fuchsin for 1 min and finally washed and air dried. Slides were examined under oil immersion.

In addition to the above staining procedure, representative strains were also examined by electron microscopy. Cells were harvested by centrifuging 18 h HIB cultures and then resuspended in 1% (w/v) potassium phosphotungstate. A drop of the suspension was spread on an electron microscope carbon support film and then shadowed with gold/palladium alloy (at a nominal angle of 45^{o}). Shadowed preparations were examined under a JEM 7A electron microscope.

Oxidation-fermentation test. The medium described by Hugh & Leifson (1953) was used. Inoculated tubes were examined daily during 3 d incubation. An acid reaction at the top of the tube was recorded as oxidative, whilst acid production throughout the tube was taken as evidence of fermentation. Cultures giving an alkali reaction were recorded as "inert" towards glucose.

Acetoin production and Methyl Red test. Organisms were grown in a medium containing (% w/v) peptone(Evans), 0.5; K_2HPO_4, 0.5; glucose 0.5; pH, 7.0. After 5 d at 30^{o} the cultures were examined for acetoin by Barritt's (1936) method and the pH was tested with methyl red.

Oxidase activity. A filter paper was moistened with a few drops of Kovacs (1956) oxidase reagent (1% (w/v) tetramethyl-p-phenylene-diamine). A platinum loop was used to transfer cells from HIA slopes to the filter paper. The development of a blue colour within 10 sec was recorded as a positive result.

Acid and gas from sugars. Purple Broth Base (Difco) containing 1% (w/v) of an appropriate sugar was used. The composition (% w/v) of the basal medium was, Protease-Peptone No.3, 1.0; Bacto-Beef

Extract, 0.1; NaCl, 0.5; Bacto-Brom Cresol Purple, 0.0015; pH 6.8. Cultures were incubated for 5 d and examined for acid and gas (collected in Durham tubes).

Indole production. One ml of ether and 0.5 ml Ehrlich's reagent (p-dimethylaminobenzaldehyde, 1.0 g; absolute alcohol, 95 ml; conc. HCl, 20 ml) were added to cultures grown in 1% (w/v) Evans peptone water for 5 d. A pink/red colouration of the solvent layer indicated the presence of indole.

Growth at 0 and 37°. HIB cultures were incubated at 0° (in a refrigerator, see page 11) and 37° (in a thermostatically controlled water bath) and examined for growth (turbidity) at 7 and 14 d.

Urease production. Christensen's (1946) urea medium was used to detect urease activity. Inoculated slopes were examined daily for up to 5 d.

MICROCOCCUS

Methods for oxidase and V-P test were as described for the Enterobacteriaceae. For other tests, the methods recommended by Baird-Parker (1966) were used. HIB or HIA cultures grown for 18 h at 30° were used as inocula, 30° being the incubation temperature for all tests.

Oxidation-fermentation test. The medium and methods used were those proposed by the International Sub-committee on Staphylococci and Micrococci (1965). The medium contained (% w/v), Difco tryptone, 1.0; Difco yeast extract, 0.1; glucose, 1.0; bromocresol purple, 0.004; Difco agar 0.2; pH 7.2. Duplicate tubes (6 x $\frac{1}{2}$") were heavily inoculated with a long wire loop, and one tube of each pair overlayed with sterile liquid paraffin. Tubes were incubated for 5 d.

Phosphatase. Phenolphthalein diphosphate agar (Baird-Parker, 1966) was inoculated and incubated for 6 d. Phosphatase activity was detected by inverting the agar plate over a Petri-dish lid containing 0.880 sp. gr. ammonia; phosphatase-producing colonies turned deep pink when exposed to the ammonia vapour.

Acetoin production. The medium used contained (% w/v) Tryptone (Difco), 1.0; Lab-Lemco (Oxoid), 0.3; yeast extract (Difco, 0.1; glucose 2.0; pH 7.2. Inoculated media were incubated for 14 d and then tested for acetoin by Barritt's (1936) method.

Acid production from sugars. Plates of basal medium (Baird-Parker, 1966) containing 5 ml of a 10% (w/v) Seitz-filtered solution of arabinose, lactose, maltose or mannitol were inoculated (4 isolates per plate) and examined daily for acid production for up to 7 d.

YEASTS

Incubation temperature for all tests was 25°, and the identification methods used were based mainly on those described by Lodder & Kreger Van Rij (1952).

Ascospore formation. Cultures were grown for 3 d on modified Gorodkowa's agar which contained (% w/v) glucose, 0.1; Evans peptone, 1.0; NaCl, 0.5; agar, 3.0; pH 7.2. Wet preparations were then examined for the presence of ascospores. Weekly examination was continued for up to 4 weeks incubation.

Pseudo-mycelium. Agar slides were prepared by allowing a drop of liquefied Potato Glucose Agar to solidify on a clean sterile microscope slide. The agar was inoculated with the yeast culture and covered with a coverslip. After 2 d incubation the slide cultures were examined for pseudo-mycelium formation.

Gas production from sugars. The following sugars were tested; glucose, galactose, sucrose, maltose and lactose. The medium used contained (% w/v) yeast extract, 0.5; sugar, 0.2. Inoculated tubes were incubated for up to 10 d and examined for the presence of gas (collected in Durham's tubes).

Carbon assimilation. The sugars tested were as noted in the above test. Two methods were used; (a) the auxanographic technique of Lodder & Kreger Van Rij and (b) the method described by Ross & Morris (1965).

(a) Plates containing a carbohydrate free basal medium (Lodder & Kreger Van Rij, 1952) were seeded with 2 ml of a heavy suspension of the yeast culture under test. Drops of sugar solutions (10% w/v) were then added on to the surface of the medium. Plates were examined for growth after 2 d incubation.

(b) 1.5 ml of a 10% (w/v) sugar solution was added to 13.5 ml of carbohydrate free medium. Plates were inoculated (six isolates/plate) using a Pasteur pipette. Control plates of basal medium were also inoculated. After 3 d and 1 week incubation the plates were checked for growth.

Fat splitting. Beef suet was melted, filtered and sterilized. 1.5 ml of the fat was poured into a warm sterile Petri-dish to give a thin, even layer. Gorodkova's agar containing 0.1% $CaCO_3$ was then poured on top of the fat layer. Plates were incubated for 5 d and a positive result was indicated by the presence of precipitated calcium salts under the inoculation streak.

Production of "starch-like" compounds. Cultures were grown for 2 weeks on the synthetic medium described by Lodder & Van Rij (1952). The plates were then flooded with Lugols iodine to reveal the presence

of starch compounds.

Nitrate assimilation. The tests were made in the liquid medium suggested by Lodder & Kreger Van Rij; KNO_3 (0.078% w/v) was the nitrogen source.

LACTIC ACID BACTERIA

Cultures were maintained on APT agar slopes containing 1.0% (w/v) $CaCO_3$ and the incubation temperature was 20° unless otherwise stated.

Gas production from glucose. To distinguish between homofermentative and heterofermentative strains, the medium of Gibson & Abd-el-Malek (1945) was used. Tubes were sealed with agar and examined for gas production during 14 d incubation.

Acid from carbohydrates. Sugar solutions were incorporated as 1.5% (w/v) in MRS basal medium (de Man, Rogosa & Sharpe, 1960). The inoculated medium was incubated for 14 d.

Ability to grow on APT agar containing 0.04% w/v potassium tellurite (APTT agar). Cultures were streaked on APTT agar and examined for growth and tellurite reduction after 48 h incubation.

Dextran production. Tetrazolium thallous acetate sucrose agar (Cavett, Dring & Knight, 1965) was used. Colonies developing on this medium were examined for "guminess".

Ammonia from arginine. The method described by Cavett (1963) was used.

ACINETOBACTER

All cultures were incubated at 20° unless indicated otherwise. Oxidase and oxidation-fermentation tests were as given for Enterobacteriaceae.

Arginine utilization. Thornley's (1960) medium in screw cap bottles

(5 ml capacity) was stab inoculated and then overlayed with sterile liquid paraffin. Bottles were incubated for 8 d and an alkaline reaction was recorded as a positive result.

Gluconate utilization. Two media were used in this test.

a) Haynes' (1951) medium which contained (% w/v) tryptone, 1.5; yeast extract, 1.0; K_2HPO_4, 1.0; potassium gluconate, 4.0; pH 7.0.

b) Paton's (1959) medium which contained (% w/v) $NH_4H_2PO_4$, 0.1; KCl, 0.02; $MgSO_4.7H_2O$; potassium gluconate 0.5; pH 7.0.

Both media were inoculated with every strain. Cultures were incubated for 10 d and then tested for the presence of reducing compounds with Benedict's qualitative reagent.

H_2S production. Clarke's (1953) medium containing 0.01% (w/v) cysteine hydrochloride was dispensed in McCartney bottles. After inoculation a small strip of sterile lead acetate paper was inserted into the mouth of the bottle. Blackening of the paper was recorded as a positive result.

Litmus milk reaction. Litmus milk medium (Oxoid) was inoculated and incubated for 14 d. Interpretation of the results was as recommended by Cowan & Steel (1965).

Growth at 0^o and 37^o. Inoculated HIB medium was incubated for 14 d at 37^o or 0^o and checked for growth (turbidity).

Detection of cytochrome. Cells grown in HIB (on a rotary shaker) for 18–24 h were harvested by centrifugation. The pellet of cells was placed on a microscope slide and examined with a microspectroscope. Cells held at liquid nitrogen temperature (Keilin & Hartree, 1949) were also examined. Under these conditions, cytochrome bands are both sharpened and intensified.

PSEUDOMONAS

Methods for oxidase and oxidation–fermentation tests and flagella staining were the same as those used in the characterization of Enterobacteriaceae. Methods used for arginine and gluconate utilization and cytochrome determination were as given above for Acinetobacter, 20^{o} incubation was used throughout.

Pigment production. King, Ward & Raney's (1954) A and B media were used for the detection of pyocyanin and fluorescein respectively. Since the formation of these pigments is enhanced by highly aerobic conditions, inoculated slopes in McCartney bottles were incubated (3 d) with the caps loose.

Production of extracellular hydrolases. Frazier's (1926) method for gelatinase; Fryer, Lawrence & Reiter's (1967) technique for lipase, and Cowan & Steel's (1965) method for the detection of starch hydrolysis were used.

Growth at 0^{o} and 41^{o}. Inoculated HIB incubated at 0 and 41^{o} for 2 weeks were examined for growth.

Utilization of organic compounds as sole carbon and energy source. All strains were tested for their ability to use the following organic compounds as sole sources of carbon and energy;

a) Carbohydrates and sugar derivatives: D–ribose, D–fucose, L–rhamnose, D–arabinose, L–arabinose, maltose, D–glucose, D–mannose, D–galactose, D–fructose, sucrose, trehalose, cellobiose, lactose, salicin, inulin, gluconate, 2–ketogluconate, mucate.

b) Fatty acids: acetate, propionate, butyrate, isobutyrate, valerate, isolvalerate, caproate, heptanoate, caprylate, caprate, pelargonate.

c) Dicarboxylic acids: oxalate, malonate, succinate, maleate, fumarate, glutarate, adipate, pimelate, suberate, azelate, sebacate, eicosanedioate.

d) Hydroxyacids: D-malate, D(-)-tartrate, meso-tartrate, DL-β-Hydroxybutyrate, DL-lactate, glycollate, glycerate, hydroxymethylglutarate.

e) Miscelaneous organic acids: citrate, α-ketoglutarate, pyruvate, aconitate, laevulinate, citraconate, itaconate, mesaconate,

f) Polyalcohols and glycols: erythritol, mannitol, sorbitol, meso-inositol, adonitol, glycerol, ethyleneglycol, propyleneglycol, 2,3-butyleneglycol.

g) Alcohols: methanol, ethanol, n-propanol, isopropanol, n-butanol, isobutanol, geraniol.

h) Non-nitrogenous aromatic and other cyclic compounds: D-mandelate, benzoylformate, benzoate, p-hydroxybenzoate, phthalate, terephthalate, phenylacetate, phenylethanediol, naphthalene, phenol, quinate, testosterone.

i) Aliphatic amino acids: glycine, D-alanine, β-alanine, DL-serine, L-threonine, DL-leucine, L-isoleucine, L-valine, L-aspartate, L-glutamate, L-lysine, DL-arginine, L-ornithine, DL-citrulline, L-amino-butyrate.

j) Amino acids and related compounds containing a ring structure: L-histidine, L-proline, L-tyrosine, L-phenylalanine, D-tryptophan, kynurenate, anthranilate, p-aminobenzoate.

k) Amines: methylamine, ethanolamine, benzylamine, putrescine, spermine, histamine, butylamine, α-amylamine.

l) Miscellaneous nitrogenous compounds: betaine, sarcosine, creatine, hippurate, pantothenate, acetamide, nicotinate.

The test substrates were sterilized by sintered glass filtration and added to the standard mineral base of Stanier, Palleroni & Doudoroff (1966) to give a final concentration of 0.1%(w/v) (except the sugars which were used at a concentration of 0.2% (w/v)). The replica plating techniques of Lederberg & Lederberg (1952) were used to screen the isolates. Each master plate (nutrient agar) inoculated with 20 different strains was used for 10 replications i.e. 9 test substrates and a terminal nutrient agar plate. Inoculated plates were scored for growth after 2 and 3 d incubation.

MICROBACTERIUM THERMOSPHACTUM

Cultures (18 h) on HIA were used as the inoculum for all tests and incubation was at 20^{o} unless otherwise stated. Methods and materials for detection of gas from glucose was as given for lactic acid bacteria. For oxidation-fermentation test the medium was as used for Micrococcus. Voges-Proskauer and MR tests were as described for Enterobacteriacea.

Growth at 0 and 37^{o}. HIB cultures incubated at 0 and 37^{o} were examined for growth after 2 d and 1 week's incubation respectively.

Thermal resistance. A known volume (0.2 ml) of 18 h HIB cultures was added to 20 ml of a sterile 10% (w/v) skim milk (Oxoid) solution. The skim-milk suspension was then dispensed in 2 ml amounts in 12 x 75 mm test tubes, ensuring that none came into contact with the upper walls of the tube. The inoculated tubes, 8 for each organisms, were plugged with cotton wool and held in a thermostatically controlled water bath. At suitable time intervals depending on the organism, 4 tubes were rapidly transferred to iced water and the number of organisms surviving determined by surface plating techniques on Milk Agar (Oxoid) medium. Decimal reduction values were calculated using

the methods of Katzin, Sandholzer & Strong (1943) using the formula:

$$D = \frac{t_2 - t_1}{\log_{10} N_1/N_2}$$

Where D = decimal reduction time (minutes)

N_1 = number of viable organisms at time t_1

N_2 = number of viable organisms at time t_2

Benzidine test (Deibel & Evans, 1960). Benzidine dihydrochloride solution followed by H_2O_2 (5% w/v) was added to colonies on HIA or APT agar after incubation at 20 or 30°. The rapid development of a blue colour was taken as evidence of the presence of iron-porphyrin compounds. The benzidine reagent was prepared according to the method of Bing & Baker (1931). Benzidine dihydrochloride (1 g) was partially dissolved in 20 ml of glacial acetic acid, 30 ml of distilled water were added and the solution heated gently, cooled and 50 ml of ethyl alcohol (95% w/v) were added.

Nitrate reduction. Cultures in a medium containing (% w/v) peptone (Evans), 1.0; Na_2HPO_4, 0.5; Lab-Lemco, 0.3; yeast extract, 0.1; glucose, 0.1; $NaNO_3$, 0.02; pH 7.2, were tested after 5 days for the presence of nitrite, and if negative, for nitrate (Cowan & Steel, 1965).

NADH oxidase activity. Cells were harvested by centrifugation (20,000 g for 15 min) of 48 h APT broth cultures (rotary shaken) grown at 20 or 30° and washed three times with 0.03M-phosphate buffer pH 7.2. Cell free extracts were prepared by disrupting the bacteria in a Braun disintegrator (Shandon, London) and removing cell debris by centrifugation. The effect of cyanide on NADH oxidase activity of the cell extract was determined by measuring

Δ 340 mμ in a Unicam SP 500 (Unicam Instruments, Cambridge, England). Quartz cuvettes (3.0 ml) with 1 cm light path were used. For basic activity, the reaction mixture contained, 0.03 M PO_4 buffer (pH 7.4), 2.7 ml; cell free extract, 0.2 ml; 2 mM nicotinamide-adenine dinucleotide (reduced), 0.1 ml. For measuring cyanide sensitivity the cuvette contained, buffer, 2.6 ml; 100 mM KCN, 0.1 ml; extract, 0.2 ml; 2 mM $NADH_2$, 0.1 ml.

Detection of cytochrome. Cells were harvested as for NADH oxidase. Whole cells were examined at room temperature or at liquid N_2 temperature using a microspectroscope. For low temperature transmittance measurements a Beckman DB spectrophotometer was fitted with a liquid N_2 reservoir which maintained the cell suspension at ~ -160^{o} (unpublished work of Dr. J. Keilin). For all observations at low temperatures cells were suspended in 50% aqueous glycerol. For reflectance spectrometry a Unicam SP 800 was fitted with a SP 890 diffuse reflectance unit.

Iron and haematin content of APT and HI media. Iron was assayed with bathophenanthroline (Smith, McCurdy & Diehl, 1952) and haematin by the haemochromogen test (Hartree, 1955).

Fermentation products from glucose. Medical flats (4 oz) were filled to the neck with the medium of McLean & Sulzbacher (1953). The sterile medium was steamed for c. 20 min, cooled to room temperature and then inoculated immediately. After 4 weeks at 20^{o}, the medium was centrifuged and the supernatant examined. The Munson-Walker technique (Triebold & Aurand, 1963) and Barker & Summerson's (1941) method were used to estimate residual glucose and lactic acid, respectively. In addition, control sterile medium and the supernatant liquid from the cultures were analysed on a Pye Model

104 gas chromatograph fitted with flame ionization detectors (W.G. Pye & Co., Cambridge). Mixtures were separated in a nitrogen flow of 90 ml/min at 110° on a column (4 ft x 5 mm ID) containing Tween 80 (15% w/v) on Chromosorb W (60–80 mesh, Johns-Manville Co.Ltd., London) pretreated with phosphoric acid.

Cis-aconitase activity. Activity was determined by measuring Δ 240 mμ in a Unicam SP 800. The reaction mixture contained 2.9 ml 0.03M-trisodium citrate in 0.05M phosphate buffer pH 7.4; 0.1 ml cell extract (prepared as described above).

Agar slide cultures. A phase contrast microscope (Zeiss photomicroscope) was used to follow the growth of isolated cells on the surface of either APT, HIA or plate count agar (PCA). Drops of medium were allowed to set on sterile slides, inoculated and sealed with a coverslip. To prevent evaporation the inoculated slides were placed in a glass chamber securely clamped to the microscope stage. Photographs were taken on 35 mm Ilford FP3 film.

Electron microscopy. Cells were harvested by centrifuging Heart Infusion Broth cultures, or from suspensions prepared from agar cultures. These were twice washed with 0.03 M Sorensen's phosphate buffer (pH 7.2) and then fixed with glutaraldehyde and osmium tetroxide by the method described by Hamilton & Stubbs (1967). The fixed cells were washed in 3 changes of distilled water. The cells were recovered by centrifuging and resuspended in a small quantity of liquefied agar (at c.50°). This was then cut into small pieces (0.5 mm^3) and dehydrated by passage through increasing concentrations of ethanol, and finally embedded in Araldite (Glauert & Glauert, 1958).

An LKB Ultratome III microtome (LKB, Stockholm) was used to cut thin sections of c 700 Å thickness. These were stained with 1% (w/v) uranyl acetate in water and lead citrate (Reynolds, 1963), and examined in a JEM 7A electron microscope (Delvijem, Finchley, London). Fixed cells on carbon coated electron microscope specimen grids were shadowed at a nominal angle of 45^{o} with a gold/palladium alloy. These were examined in a JEM 6A electron microscope fitted with an ACW low magnification device.

Cells were also examined by freeze etching. A specimen of cells was placed on to a small copper cap and then immersed in liquid Freon 22 (Monochlorodifluoromethane) held at its freezing point of -150^{o} with liquid nitrogen. The specimen and cap were rapidly transferred to the vacuum chamber (Polaron Ltd., London) and held at -180^{o}. After evacuation (c 3×10^{-6} torr) the temperature was raised to -100^{o}, the specimen fractured, and the surface ice allowed to sublime. A platinum/carbon replica of the etched surface was prepared and examined under the electron microscope.

RESULTS AND DISCUSSION

The scheme used for the preliminary separation of microorganisms isolated in the present study is given in Table 2.

Representative strains from the following were studied in greater detail, Enterobacteriaceae, Micrococcus, Yeasts, lactic acid bacteria, Acinetobacter, M. thermosphactum. The taxonomy of these organisms and their isolation by other workers is discussed.

TABLE 2

PRELIMINARY IDENTIFICATION OF MICROORGANISMS ISOLATED FROM BEEF*

ORGANISM	Gram Reaction	Morphology	Flagella	Catalase	Oxidase	Utilization of Glucose	NH_3 from Arginine
Pseudomonas	–	rod	polar	+	+	O	+/–
Acinetobacter	–	coccobacillus	nm	+	+/–	I	–
Aeromonas	–	rod	polar	+	+	F	+/–
Enterobacteriaceae	–	rod	peritrichous	+	–	F	n.t.
Microbacterium	+	pleomorphic rod	nm	+	–	F	n.t.
Lactic Acid Bacteria	+	coccus/rod	nm	–	–	F	n.t.
Staphylococcus	+	coccus	nm	+	–	F	n.t.
Micrococcus	+	coccus	nm	+	+/–	O/I	n.t.
Bacillus	+	rod, spore-forming	peritrichous	+	–	n.t.	n.t.
Yeasts	+	budding, ellipsoidal cells, mycelium	nm	+	n.t.	n.t.	n.t.

* For details of Methods for tests see text.

+, All strains positive; –, all strains negative; +/–, positive or negative; O, Oxidative; F, Fermentative; I, Inert; nm, non motile; n.t., not tested.

ENTEROBACTERIACEAE

One hundred isolates were examined. For reliable generic identification of the Enterobacteriaceae it is necessary to carry out a wide range of tests on a large number of isolates (Edwards & Ewing, 1962). Although this was not done in the present work, it was possible to recognize two main groups (Table 3). Organisms of group A were tentatively identified with Enterobacter/Hafnia (Edwards & Ewing, 1962) and those in Group B appeared to be related to Proteus.

Enterobacteriaceae do not normally form significant proportions of the microbial association developing on chilled meat. Early observations that they were important in deterioration should be treated with caution. Proteus gained an unwarranted status as the principal component of the spoilage flora (Weinzirl, 1924). But, as pointed out by Haines (1937), inappropriate methods (e.g. 37^{o}incubation) were used in many such studies. Thus if viable count plates from meat stored at 0-5^{o} are incubated at this temperature, it is probable that only mesophiles will grow and that erroneous conclusions would be drawn regarding the major microbial groups of the association. Likewise, with meat stored at 37^{o} the flora will be different from that stored at 0-5^{o}, and the results obtained at 37^{o} should not be extrapolated to lower temperatures. It was shown in the present study (Fig.7) that Enterobacteriaceae, although often present in significant numbers at the beginning of storage, were rapidly overgrown and after a few days were not normally detectable on total count plates.

Although these organisms were capable of growth at 0^{o} (Table 3), to demonstrate their presence it would seem necessary to use selective media as shown by Gardner (1965).

The occurrence in meat of cold tolerant Enterobacteriaceae has been long recognized (Haines, 1937). In later studies (Eddy & Kitchell, 1959) the properties of such organisms from various meats were examined. Strains (28) able to grow well at -1.5^{o} were investigated, the following genera being represented, Citrobacter, Klebsiella, Aerobacter (Enterobacter) and Hafnia. It is of interest that c 70% of the strains were identified with the Aerobacter (Enterobacter) - Hafnia group, a result which is similar to that reported here (Table 3). Organisms of this group were also shown to be the principal representatives of the Enterobacteriaceae occurring in prepacked pork (Gardner, Carson & Patton, 1967). These authors noted that with samples stored at 16^{o} for 4 d Enterobacter - Hafnia comprised on average 34% of the flora. In comparable samples stored for 14 d at 2^{o} they were present at levels corresponding to 1% of the flora. However, this figure was calculated on the basis of the flora of 1 sample out of 10 comprising 11% Enterobacter-Hafnia, none being detected from the remaining 9 samples. Non-lactose fermenting Enterobacteriaceae have also been isolated from stored poultry meat (Barnes & Thornley, 1966). In these studies chickens were held at either 1, 10 or 15^{o} until off odours were detected. At the highest temperature, 27% of the flora consisted of Enterobacteriaceae most of which resembled Enterobacter. Surprisingly, at 1^{o} these organisms were present at 3% after 10-11 days storage. This result was derived from what

appears to be insufficient data, only 40 isolates were identified and the 3% represents one strain only.

There are several examples noted in the literature of cold-tolerant Enterobacteriaceae growing in milk or milk products held at 5° (Thomas, 1958; Witter, 1961). For example, detailed studies of the psychrophilic flora of commercial dairy products stored at 4° (Schultze & Olson, 1960a, b) showed that c 11% of the isolates examined were coliform bacteria, the majority of which were identified with Aerobacter (Enterobacter) cloacae.

The present study as well as those of previous investigations suggest that cold tolerant coliform organisms are common contaminants of chilled meat but that unlike milk, their contribution to the microbial association is negligible. Nevertheless, routine examination for such organisms has been recommended (Mossel, 1968) since they may indicate poor hygiene at some stage of production.

TABLE 3

PROPERTIES OF ENTEROBACTERIACEAE ISOLATED FROM BEEF

Property	Group A	Group B
Gram strain	-	-
Flagella	peritrichous	peritrichous
Oxidation/Fermentation test	F	F
Gas from glucose	+ (64)*	+
Acid from lactose	-	-
Oxidase	-	-
Catalase	+	+
Acetoin	+ (82)	- (5)
Methyl red test	-	+
Urease	-	+
Indole	-	+
Growth at 0^{o}	+ (80)	+ (6)
Growth at 37^{o}	+	+
Number of isolates	92	8

+, all strains positive; -, all strains negative; F, fermentative.

*, number of strains giving the reaction noted.

MICROCOCCUS

A representative selection (50 strains) isolated during the course of this work were studied. They were identified (Table 4) with the scheme proposed by Baird-Parker (1963, 1965, 1966).

It will be seen from Table 4 that strains of sub-group 5 were the most common. Baird-Parker (1965), who studied the properties of Micrococcus from foods and other sources, found that the majority of the strains isolated from meat products and bacon were members of sub-group 5. Further investigations of the flora of meats and meat products have also demonstrated the frequent occurrence of sub-group 5 (Dr. A.C. Baird-Parker, pers. comm.). Gardner (1965) found that mannitol non-fermenting types (presumably sub-groups 1, 2 & 8) comprised c 80% of the micrococci isolated from ground beef stored for 4 d at 15^{o}.

As with the Enterobacteriaceae, Micrococcus commonly occur in high proportions of the flora of fresh meat. On total count plates obtained from slimy beef stored for 10 d at 0^{o}, it was shown that when plates were incubated at 37^{o}, 40% of the flora were micrococci (Haines, 1933a). With incubation at 20^{o} micrococci were not detected. The reason for these results is due to the small proportion of organisms growing at 37^{o} (i.e. total count, 1.25×10^{4} bacteria/square cm) as compared to that at 20^{o}(i.e. total count, c 10^{10} bacteria/square cm). A possible source of the micrococci was suggested (Haines, 1933c). It was shown that these organisms could contribute up to 40% of the air microflora in slaughterhouses.

Later (Empey & Scott, 1939) studied the contamination acquired in meat works and reported that of the organisms viable at -1°, 7% were Micrococcus. More recent investigations have confirmed these observations. Proportions of micrococci expressed as % of the initial flora have been given as 50% (Rogers & McCleskey, 1957) or 10% (Corlett, Lee & Sinnhuber, 1965). During storage of beef at temperatures in the range 0-5° their proportions rapidly decreases and in Rogers & McCleskey's studies this had fallen to 5% after 14 d storage. Essentially the same results have been obtained with pork (Gardner et al., 1967) and chicken (Barnes & Thornley, 1966). In the investigations on pork the level of micrococci was as much as 50% initially, but after storage for 14 d at 2° they were not isolated from total count plates. With chickens taken from a processing line, the starting level was again 50%. After 10-11 d at 1° when spoilage was manifest, cocci were not isolated, a result which agrees with the earlier work by Walker & Ayres (1956).

The foregoing studies are in agreement with those obtained in the present work, thus it can be seen that Micrococcus comprised c 10% of the initial flora but were not detectable after a few days storage at 4°(Fig. 4,7).

The change in the numbers and types of organisms during the period from slaughter through to display at retail has been followed (Stringer, Bilskie & Naumann, 1969). After slaughter 37% of the flora were Micrococcus. This figure rose to 47% after storage in the chill room (c 4°)a finding which, as the authors state, is contrary to what would be expected. Before shipment the Micrococcus

was around 40% and finally, at retail, they were undetectable.

If the storage temperature is increased then these organisms can become numerically more important. Thus Ayres (1960), who made a detailed study of the effects of temperature on the microflora of beef steaks found that at 15^{o} or above, Micrococcus and Pseudomonas became the dominant types. A more recent study of the changes in flora of beef (Gardner, 1965), was limited to temperatures $\leqslant 15^{o}$. However even at 15^{o} Micrococcus were not present in sufficient numbers to be isolated (i.e. c 0.1% of the spoilage flora).

The occurrence of Micrococcus in meat products (Kitchell, 1962) will not be considered here, but it should be borne in mind that in these foods, Micrococcus can be the most important contaminant.

The question arises as to whether Micrococcus, able to grow at low temperature, should be considered psychrophilic although there are several reports of members of these organisms multiplying in the range $0-5^{o}$ (Bedford, 1933; Gorovitz-Wlassova & Grinberg, 1934; Thomas, 1958; Witter, 1961). According to accepted definitions (Ingraham & Stokes, 1959) they should not be termed psychrophilic. One possible exception is Micrococcus cryophilus (McLean, Sulzbacher & Mudd, 1951). Current opinions, however, would tend to exclude this organism from the genus Micrococcus, and in the next edition (8th) of Bergey's Manual it will be placed in the appendix (Dr. A.C. Baird-Parker, pers. comm.). The reasons for this decision were based on differences in DNA base ratio (Bohačeк, Kocur & Martinec, 1967) and cell wall structure (Mazanec, Kocur & Martinec, 1966).

TABLE 4

GROUPS OF MICROCOCCUS ISOLATED FROM BEEF

Property	Sub-Group						
	1	2	3	4	5	6	8
Phosphatase test	-	-	-	-	-	+	-
Acetoin production	+	+	v	+	-	-	-
Acid from arabinose	-	-	-	+	+	+	-
lactose	-	+	v	+	+	+	-
maltose	+	+	+	+	+	+	+
mannitol	-	-	+	+	+	+	-
Number of isolates	2	12	9	1	22	1	3

+, positive; -, negative; v, variable.

YEASTS

The strains studied (100) were isolated on Plate Count Agar acidified to pH 3.5 with citric acid. The representative selection obtained from various experiments during the present investigation. They were characterized by the methods described by Lodder & Kreger - Van Rij (1952). Table 5 summarises the results obtained. It will be noted that sporogenous yeasts were not isolated. This agrees with previous studies on beef (Ayres, 1960), the types isolated being *Candida*, *Torulopsis* and *Rhodotorula*.

The genera and species listed in Table 5 have been shown by others to occur on fresh beef, poultry and other protein foods. Among the earliest investigations, Empey & Scott (1939) made a comprehensive study of the microbial contamination acquired in meat works. Using Czapeck's agar (pH 3.5) they showed that yeasts formed a significant proportion of the organisms present on beef post-slaughter and able to grow at -1^{o}. The main genera of yeasts isolated were *Candida* and *Geotrichoides*. It is interesting to note that one of the *Candida* strains was subsequently named after Scott (i.e. *C. scottii*) and that this particular organism was isolated in the present study (Table 5).

When following the changes in numbers and types of yeast in chilled beef, it is necessary to use selective media. Presumably because of their comparitively long generation time at low temperature ($0-5^{o}$), yeasts are normally overgrown by bacteria. For example, yeasts could comprise 20% of the initial flora of pork but they were not isolated from total count plates after storage for 14 d at 2^{o}

(Gardner et al., 1967). Similarly, in an earlier study (Gardner, 1965) on ground beef, numbers of yeasts represented only a small fraction of the microflora during storage. Thus when the total viable count was c 10^9 organisms/g, the count on malt agar was $<10^5$/g. Rogers & McCleskey (1957) have shown that yeasts may be present initially at levels of > 40% of the flora but, after storage at 7° for 14 d, none were detected. Similar results were found in the work reported here; total numbers of yeasts usually represented about .001% to .01% of the microbial populations. Furthermore packing beef in CO_2:O_2 mixtures apparently did not affect these proportions.

With antibiotic treated poultry meat, it has been shown that yeasts assume a much greater significance in terms of numbers and proportions. Ayres, Walker, Fanelli, King & Thomas (1956) compared the flora developing on treated (10 ppm chlortetracycline) and untreated chicken meat. After 12 d at 4.4°, yeasts accounted for c 5% of the flora of treated and < 0.001% of control meat. In the same year, it was reported (Walker & Ayres, 1956) that yeasts comprised c 5% of the flora of untreated poultry meat (storage 12 d at 4.4°), a percentage which does not agree with that derived from estimates of yeast numbers. The authors found that at 12 d the total viable count was c 10^9/microorganisms per cm^2. At this time the yeast count was given as $10^5/cm^2$, which represents only 0.01% of the flora. In a later paper (Walker & Ayres, 1959) yeasts (39 strains) derived from stored poultry were characterized in detail, and the following identified: Trichosporon, Torulopsis, Candida and Rhodotorula. It is noteworthy that Torulopsis famata, T. inconspicua and C. scottii were relatively common (see Table 5). The yeast fraction on antibiotic treated

poultry was also studied by Njoku-obi, Spencer, Sauter & Eklund (1957) and Wells & Stadelman (1958). The former authors identified 5 genera (Saccharomyces, Torulopsis, Geotrichum, Rhodotorula & Candida) of which S.cerevisae was the most numerous. Wells & Stadelman found only 3 genera, Rhodotorula, Cryptococcus and Torulopsis.

Irradiation of poultry can also result in a flora to which yeasts make a significant contribution. Thornley, Ingram & Barnes (1960) showed in irradiated chickens (250,000 rads) yeasts comprised 90% of the flora. This was attributed to the high resistance of yeasts as compared with Pseudomonas etc. During storage (1^{0}) of treated samples, the Pseudomonas/Achromobacter (Acinetobacter) complex became predominant. With irradiated beef a different spoilage flora develops (Wolin, Evans & Niven, 1957; Thornley & Sharpe, 1959) in which M. thermosphactum is dominant.

Some interesting recent work (Dr. T.A. Roberts, pers. comm.) on irradiated vacuum packed beef, pork, lamb, veal & venison has shown that the spoilage flora consisted almost entirely of yeasts (80-95%).

TABLE 5

PROPERTIES OF YEASTS ISOLATED FROM BEEF

Property	Group A	Group B	Group C	Group D
Cellular morphology	oval (2–4μx3–6μ)	oval (2–5μx3–7μ)	oval (2–4μx3–6μ)	oval/elongate (2–5μ x 5–20μ)
Ascospore formation	–	–	–	–
Pseudomycelium	–	–	–	+
Starch compounds produced	–	–	–	–
Fat splitting	–	+	–	–
Gas production from				
glucose	–	–	+	–
galactose	–	–	+	–
sucrose	–	–	+	–
maltose	–	–	–	–
lactose	–	–	–	–
Carbon assimilation,				
glucose	+	+	+	+
galactose	–	+	+	+
sucrose	–	+	+	+
maltose	–	+	+	+
lactose	–	–	–	–
Nitrate assimilation	–	–	+	+
Relative occurrence (%)	37	15	22	26
Possible identity	Torulopsis inconspicua	Torulopsis famata	Torulopsis versatalis	Candida scottii

+, positive; –, negative.

LACTIC ACID BACTERIA

Some properties of 200 strains were determined, they were Gram positive, catalase negative, non-sporing, non-motile short rods or cocci. Half the strains (Group A) were originally recovered on Rogosa's agar which was used to estimate numbers of lactic acid bacteria in these studies. The others (Group B) were recovered on Plate Count Agar from meat stored (-1^{o}) in 80% O_2: 20% CO_2 (Fig. 9).

The schemes of Garvie (1967), Cavett (1963) and Sharpe, Fryer & Smith (1966) were used in an attempt to identify the strains.

Group A (organisms recovered on Rogosa's agar)

Some (33%) of the strains were heterofermentative cocci, did not grow on APTT agar, did not produce NH_3 from arginine, and were identified with Leuconostoc (Cavett, 1963). Garvie (1960) recognized 6 groups within Leuconostoc. In a subsequent report (Garvie, 1967)this scheme was modified and the following species defined: L. mesenteroides, L. dextranicum, L. paramesenteroides, L. lactis, L. cremoris and L. oenos. They were separated mainly on production of slime from sucrose, acid formation from sugars and growth in various media. The strains isolated in the present work were identified with L. mesenteroides; they produced dextran, grew at 37^{o} and gave an acid reaction from trehalose, sucrose, xylose, cellobiose, lactose, arabinose and mellibiose and a weak acid reaction with salicin.

The remaining strains (67%) were homofermentative rods which grew well on APTT agar. They were identified with the Streptobacterium group of Lactobacillus. Since they did not produce acid from mannitol, they were considered to be similar to the atypical streptobacteria which Cavett (1963) isolated from vacuum packed bacon.

Group B (organisms recovered on Plate Count Agar)

The strains (100) were identified with Leuconostoc and were the predominant microbial type on beef stored at -1^{o} under 80% O_2 + 20% CO_2 (Fig. 9). Their biochemical and morphological properties with two notable exceptions, were similar to Leuconostoc mesenteroides. This organism is normally considered capable of growth at 37^{o} (Garvie, 1967), a feature which was lacking in our isolates. The reason for this result is not known. It may well be that low temperature (-1^{o}) and gas packing selected unusual psychrophilic strains of this species. The other difference was their failure to grow on selective media, e.g. Rogosa's agar and Keddie's (1951) agar. This was associated with their inability to tolerate acetate at low pH. It is of interest that Dr. G.A. Gardner (pers. comm.) isolated leuconostocs from vacuum-packed bacon burgers which did not grow on acetate agar. The majority of Gardner's strains produced dextran from sucrose but differed in some respects from the organism isolated in the present work, for example they failed to produce acid from melibiose.

In the experiments reported here, lactic acid bacteria became dominant only in gas-packed beef stored at -1°. Under all other conditions tested, they comprised only a small fraction of the flora. Although their numbers increased, they formed only 0.01% of the spoilage microflora. In general these results agree with previous investigations. For example, in a study of the effects of film permeability (Jaye et al., 1962) it was shown that with ground beef packed in cellophane (gas permeable; storage -1.1°/ 12 d) lactic acid bacteria comprised c 0.01% of the total count. With pork wrapped in permeable film and stored for 4 d (16°) the flora contained 1% lactobacilli (Gardner et al., 1966). This is a higher figure than would be expected, but it should be pointed out that it was calculated from the examination of the flora of one sample (out of six) which contained 7% lactobacilli.

Comparable studies were made on samples stored for 14 d at 2°. In these experiments, lactobacilli accounted for 30% of the flora, which the authors attribute to the CO_2 concentration of the internal atmosphere of the samples. Thus, a CO_2 level of c 12% appeared to inhibit the Pseudomonas - Achromobacter (Acinetobacter), permitting the growth of lactobacilli. This does not seem to be a wholly satisfactory explanation since with packs stored at 16°, CO_2 level was c 25% and yet lactobacilli comprised only 1% of the flora. Halleck, Ball & Stier (1958) also reported some puzzling results on the flora of fresh meats packed in films of various gas permeabilities. These studies were done on lamb, pork and beef stored at either 1.1-3.3° or 4.4-6.7°. The authors claimed that the permeability of the film did not selectively affect the rates of growth of the

predominant genera Pseudomonas-Achromobacter. Even with samples stored under highly impermeable films or in hermetically sealed cans the Pseudomonas-Achromobacter group were dominant. However, in this work, analysis of the flora was apparently by visual inspection only of total count plates, a procedure which would seem open to criticism. For instance colonies of lactobacilli and M. thermosphactum might easily be confused, especially when plates were incubated at 30° as at this temperature M. thermosphactum gives a negative catalase reaction (Davidson, Mobbs & Stubbs, 1968). When attempting to explain the results of Halleck and his co-workers, Cavett (1968) suggested that unsuspected diffusion of gas through the packaging materials may have occurred, or that some undefined alternative hydrogen acceptor was present in the system.

When beef is vacuum-packed or sealed in a close-fitting impermeable film, a change from the normal catalase positive, Gram negative flora to one consisting of catalase negative Gram positive organisms occurs. Jaye et al.(1962) studied the flora of beef tight-wrapped with saran (co-polymer with polyvinylidene chloride) and stored at -1.1 or 3.3°. A selective medium was used for determining the numbers of lactic acid bacteria. At 11 d storage at 3.3 these organisms accounted for half the total count. Similar results were obtained with meat vacuum-packed in glass jars (Noskova & Pek, 1963); lactic acid bacteria became predominant and towards the end of storage were present almost in pure culture.

Vacuum-packing of meat products also results in a microflora composed mainly of lactic acid bacteria. Several investigators have described the change from an aerobic flora to lactic acid

bacteria flora in these products (Cavett, 1968). One such study (Alm, Ericksen & Molin, 1961) was concerned with the effect of vacuum-packing on some sliced processed meat products, i.e. wieners, salt cured meat and German sausage) stored at various temperatures. With the salt cured meat and wieners, the initial flora consisted mainly of Bacillus, Micrococcus and Lactobacillus. After 9 d at 15° Lactobacillus had become dominant.

In summary, it can be concluded that microorganisms belonging to the lactic acid bacteria play a minor role in the spoilage of aerobically stored fresh meat. However, if the growth of the normal spoilage flora is depressed e.g. by vacuum-packing or gas (80% O_2 + 10% CO_2) packing and storage at -1°, catalase negative bacteria can become the principal component of the flora.

ACINETOBACTER

These strains (total 110) were isolated on Plate Count Agar from beef stored under various conditions. They were characterized by the methods described by Thornley (1967). The following properties were common to all strains: Gram negative (see below), non-sporing, non-motile, catalase positive, coccal rods, occurring singly and in pairs, negative reaction in the arginine test, "inert" in Oxidation-Fermentation test and negative reaction in the gluconate test. With the Gram-stain, these organisms frequently appeared Gram-intermediate (or variable) an observation which has been noted by others (Henriksen, 1952; Reynolds & Cluff, 1963; Thornley, 1967). The tendency to retain the crystal violet may be related to the unusual structure (Thornley & Glauert, 1968) and/or chemical composition (Gallagher, 1968) of the cell envelope.

Three groups of organisms were recognized in this study. Organisms similar to these were the subject of a detailed study using traditional methods and computer analysis of results (Thornley, 1967). She recognized a major group with three phenons (S value $>85\%$). Group A (Table 6) were similar to Thornley's phenon No. 4i which she considered would include strains of Moraxella duplex var non-liquefaciens. Organisms of Group B were similar to Thornley's phenon 4 ii except that our isolates gave a negative reaction in the gluconate test. This phenon contained no named strains. Group C strains have the same reactions as organisms which Thornley termed Moraxella lwoffi

The nomenclature and classification of microorganisms having the general properties described above have, over the years, been in a state of confusion and uncertainty. At various times they have been placed in at least 15 genera, the following being the most common: Achromobacter, Acinetobacter, Alcaligenes, Bacterium, Diplococcus, Haemophilus, Herella, Mima, Moraxella, Micrococcus and Neisseria. A summary of the literature pertaining to the nomenclature of this ill-defined group may help to clarify the origins of some of the perplexities.

Moraxella - Acinetobacter. Morax (1896) and Axenfield (1897) were apparently the first workers to recover these organisms, and they became known as the Morax-Axenfield bacillus. Later, the name Bacillus lactunatus was suggested (Eyre, 1900). A similar but less nutritionally fastidious bacterium was isolated and named Bacillus duplex (Petit, 1900). Scarlett (1916a,b) described 2 further varieties which became known as B. duplex var non liquefaciens and var josephi.

The subsequent inclusion of these in Haemophilus was questioned by Lwoff (1939), and he defined a new genus Moraxella in which two species were recognized i.e. M. lacunata & M. duplex. The following year, another species M. lwoffi was added to the genus (Audureau, 1940). A subsequent study, (Piechaud, Piechaud & Second, 1951) of strains designated M. lwoffi, revealed differences in carbohydrate utilization, and those able to produce acid were named M. lwoffi var glucidolytica. In a later paper, (Piechaud, Piechaud & Second, 1956), this variety was recognized as a separate species and the following classification of the genus was proposed.

Moraxella

Group I (oxidase positive)	Group II (oxidase negative)
M. lacunata	M. lwoffi
M. duplex	M. glucidolytica

The above classification has been adopted by some workers (e.g. Leduc et al., 1969) and was recommended for use in clinical bacteriology.

Other workers would exclude oxidase negative organisms from Moraxella. Steel & Cowan (1963) proposed that M.lwoffi together with bacteria such as Haemophilus pertussis and Bacillus mallei should be placed in Acinetobacter as defined by (Brisou & Prevot, 1954). This would include Groups B & C (Table 6). Steel & Cowan's proposal was immediately criticized by Lwoff (1964) who stated that the species lwoffi, duplex and anitrata must be retained in the Moraxella. A contrary view has been taken by others. Thus Baumann, Stanier & Doudoroff (1968 a, b), made a detailed study of the nutritional and physiological properties Moraxella group and concluded that Moraxella should be reserved for oxidase positive types, negative strains being placed in Acinetobacter. The oxidase reaction was shown to the correlated with the presence or absence of cytochrome c. This observation was confirmed in the present work, thus Group A was shown to possess cytochrome c whilst Groups B & C did not. Baumann et al.(1968 b) suggested that the type species of Acinetobacter should be A. calco-aceticus, thereby crediting

Beijerinck with the first adequate description of this organism (Micrococcus calco-aceticus, Beijerinck). Baumann et al. (1968a) favoured M. osloensis as the type species, and the genus to be comprised of osloensis and 3 other species.

The genus Acinetobacter was defined (Brisou & Prevot, 1954) for the inclusion of non-motile strains, previously designated Achromobacter. Others however have continued to use the name Achromobacter for both motile and non-motile strains. Pickett & Manclark (1965) and Gilardi (1968) considered Achromobacter lwoffi and Achromobacter anitratus to be preferable names.

Mima-Herella. De Bord (1942) created the tribe Mimeae, containing Mima, Herella and Colloides. Colloides have properties similar to Escherichia freundii and would therefore be better placed in the Enterobacteriaceae (Henriksen, 1952). The genus Mima contained the species polymorpha (oxidase negative) and an oxidase positive variety, var oxidans. Mima polymorpha has since been shown to be indistinguishable from Moraxella (Acinetobacter) lwoffi and several investigators (e.g. Henriksen, 1952; Henderson, 1965; Pickett & Manclark, 1965; Gilardi, 1968; Thornley, 1967; Baumann et al., 1968a) have recommended that the name Mima be abandoned. Likewise M. polymorpha var oxidans is closely related to and probably identical with Moraxella duplex (Henriksen, 1952, 1963). Herella is also considered to be an "illegitimate epithet" (Pickett & Manclark, 1965). It has the same properties as the following, B5W (Stuart, Formal & McGann, 1949) and Bacterium anitratum (Schaub & Hauber, 1948), Moraxella glucidolytica. This group and its various synonyms are

listed in Table 7. Although many bacteriologists would favour abolition of Mimeae, a few advocate its retention (Nelson & Shelton, 1965). Furthermore, Weed (1961) disagreed with the omission of Mimeae from the 7th edition of Bergey's Manual, because "failure to recognize Mimeae has not stopped infections caused by the organisms". It is noteworthy that medical microbiologists in general continue to use the names Mima and Herella whereas those concerned with food, for example, tend to use Acinetobacter, Achromobacter etc. This is probably one of the main causes of the confusion in nomenclature.

At present there is no widely accepted classification, and even modern techniques such as DNA base ratios (Bøvre, Fiandt & Szybalski, 1969) have not completely clarified the situation.

There are many recorded instances of organisms similar to the above being isolated from foods and other sources, and the name achromobacters was commonly used. The early work of Haines (1933) and Empey & Scott (1937) suggested that Achromobacter were responsible for the deterioration of fresh beef. Since the revised definition of Pseudomonas (Bergey's Manual 6th edition) the majority of Empey & Scott's isolates have been identified with Pseudomonas (Brown & Weidemann, 1958). More recently (Ayres et al., 1950) acinetobacter-like organisms were isolated from stored chicken and identified with Alcaligenes - the validity of this genus is in doubt (Hendrie, Hodgkiss & Shewan, 1964). It seems probable that in many of the reports on the isolation of Achromobacter from stored meats (Kirsch et al., 1952; Nagel et al., 1960; Ayres, 1960; Gardner,

1965) they would now be termed Acinetobacter. In recent studies (Barnes & Thornley, 1966; a Vanderzeat & Nickleson, 1969) the name Acinetobacter is becoming more widely used.

In reports where the possible public health significance of these organisms in foods was discussed, there is a tendency to revert to the older terminology and the use of names such as Herella, Mima etc.(Snodgrass & Koburger, 1967). They found that these organisms were present in meat and seafoods, dairy products and vegetables in 32%, 32% and 30% respectively of samples examined, and concluded that such foods act as vehicles and may represent a potential health hazard particularly to infants and the aged. Eller (1969) found that Herella vaginicola (Acinetobacter anitratus) was present in 4.7% of 364 samples of frozen foods and again the possible health danger was discussed. The importance of related (but not necessarily identical) organisms in human disease is fairly well established (Daly, Postic & Kass, 1962; Reynolds & Cluff, 1963; Alami & Riley,1966). They have been shown to be associated with bronchial infections (Henriksen, 1952; Ashley & Kwantes, 1961; Bottone & Allerhand , 1967) bacterial endocarditis, (Pike, Schulze & McCullough, 1951; Minzter, 1956), meningitis (Olafsson, Lee & Abernethy, 1958; Torregrosa & Oritz, 1961), Waterhouse-Friderichsen syndrome (Townsend, Hersey & Wilson, 1954) and fulminating septiceamia (Faust & Hood, 1949). Despite these examples, the role of these organisms in human pathogenicity is difficult to evaluate, since they apparently possess a low degree of virulence and infect hosts with altered or lowered resistance (Reynolds & Cluff, 1963).

From the food hygiene viewpoint, it is not possible at this

time to suggest what the importance of these organisms may be except that the presence of certain types in high numbers may be undesirable. Fortunately, these organisms normally represent only a fraction of the microflora of chilled foods. This may be a result of the slower growth in food where the 0_2 tension is reduced (Baumann, 1968).

TABLE 6

PROPERTIES OF ACINETOBACTER ISOLATED FROM BEEF

Property	Group A	Group B	Group C
Oxidase	+	–	–
Growth at 0^{o}	+	+	–
" " 37^{o}	–	–	+
Reduction of litmus milk	+	–(19*)	+
H_2S production	–	+	– (21)
Number of strains	61	26	23

+, all strains positive; –, all strains negative;

*, number of strains giving the reaction noted.

TABLE 7

SYNONYMS FOR THE TRIBE MIMEAE[+]

Member	Synonyms
Mima polymorpha var oxidans	Moraxella duplex*
	Moraxella nonliquefaciens
Mima polymorpha	Achromobacter lwoffi
	Acinetobacter lwoffi *
	Moraxella lwoffi
	Alcaligenes metalcaligenes
Herella vaginicola	Achromobacter anitratus
	Acinetobacter anitratus*
	Bacterium anitratum
	B5W
	Diplococcus mucosus
	Moraxella glucidolytica
	Moraxella vaginicola
	Neisseria mucosus
	Acinetobacter calco-aceticus
Colloides anoxydana	Citrobacter group*
	Escherichia freundii
	Paracolon intermedium

+ Data derived from Pickett & Manclark (1965); * Suggested designations.

PSEUDOMONAS

Strains (100) were randomly selected from a collection assembled during the course of the present study. The majority were isolated from beef which had been stored until spoilage was evident. Tests used for characterization were as discussed by Shewan *et al*. (1960) and Stanier *et al*. (1966). Properties common to all strains are shown in Table 8. Nineteen produced a green fluorescent pigment on the medium of King *et al*. (1954) medium; none formed pyocyanin, 8/19 of the pigmented and 62/81 of the non-pigmented strains hydrolyzed gelatin. Similar proportions were positive in the test for lipase (substrate, tributyrin). In accord with the results of Sherris, Shoesmith, Parker & Brekon (1959), Thornley (1960) and Stanier *et al*.(1966), all the fluorescent strains produced NH_3 anaerobically from arginine. However 16% of the non-pigmented types were negative, a finding which agrees with that of Stanier *et al*. (1966) ("this character is far from universal among the pseudomonads").

Based on the above characteristics, the achromogenic strains could be assigned to Group II, and the pigmented types to Group I *Pseudomonas* of Shewan *et al.* (1960). The non-pigmented strains appear to be most closely related to *Ps. fragi*; the proteolytic fluorescent strains were identified with *P. fluorescens* and the pigmented non-proteolytic strains with *Ps. putida*.

Stanier *et al.* (1966) published a comprehensive study of the nutritional and other properties of the aerobic pseudomonads, and

Table 9 gives the results obtained in the present study together with those of Stanier and his collaborators. It should be pointed out that with our isolates, the results for arginine dihydrolase were based simply on NH_3 from arginine (Thornley, 1960), whereas Stanier *et al*. (1966) used much more precise and stringent methods. With regard to mechanism of aromatic ring cleavage, the reagents and methods described by Stanier *et al*. (1966) were used. It was not possible to identify *orthro* or *meta* cleavage and no satisfactory explanation can be offered for this failure.

It will be seen (Table 9) that our non-pigmented isolates did not fall neatly into any of the groups recognised by Stanier *et al*. This is perhaps not unexpected since in their investigations few representatives of *Pseudomonas* responsible for spoilage of refrigerated protein foods(Group II of Shewan *et al*., 1960) were included in the study. Their strains were obtained from enrichment cultures with a single carbon and energy source, which could be expected to select specific nutritional types.

Table 10 lists the organic compounds used as sole carbon and energy source by the strains recovered from beef. There was no obvious difference of pigmented and non-pigmented strains. Selected results, together with those obtained by Stanier *et al*. are presented in Table 11. It can be seen that all beef isolates (which includes the achromogenic strains) appeared to be closely related to *Ps.fluorescens* and/or *Ps.putida*. Although on nutritional grounds no reliable separation of non-pigmented and pigmented strains was obtained, it was possible to subdivide the fluorescent types

using a simplified version of the scheme proposed by Stanier et al. These results are given in Table 12 and it will be seen that both species occurred in approximately equal proportions.

For many years the nomenclature and classification of Pseudomonas and related organisms has been controversial. Significant contributions up to the late 1950's were reviewed by Ingram & Shewan (1960). They discussed the classifications proposed by various workers and stressed how these gave rise to difficulties in defining species and genera within the group. In studies on numerous characteristics of a range of fluorescent pseudomonads (Rhodes, 1959) it was concluded that recognition of individual species was not justified. This view was later contested and stated to be invalid since the nature of Rhodes' study was such that "significant taxonomic traits were overlooked" (Stanier et al., 1966). In attempts to develop a general classification (Lysenko, 1961; Iizuka & Komagata, 1963 a, b), utilization of organic compounds as sole carbon and energy source was used as a criterion for subdivision. Again these investigators were criticized since their attempts to extend the nutritional spectrum of the pseudomonads were "at best half-hearted" (Stanier et al., 1966). It would appear that most success is likely to be achieved by investigations into the basic metabolism, such as control mechanisms for enzyme synthesis (Stanier, 1968).

Although uncertainty still surrounds the taxonomy of the pseudomonads, there is little doubt as to their cardinal importance

in the deterioration of chilled protein foods. This has been discussed in relation to fresh meat in an earlier section. It would seem that few workers have attempted to identify Pseudomonas associated with meat spoilage to species level, although several investigators have estimated proportions of pigmented and non-pigmented types. It has been stated (Kitchell, 1967) that the American workers attach more importance to pigmented pseudomonads, whereas investigations in this country have in general tended to implicate non-pigmented types. However there is apparently little evidence to support this view. Thus from both countries there are reports of either pigmented or non-pigmented being of equal importance, a situation which applies to beef and chicken. Reports in the U.S.A. that pigmented strains are more common on spoiled meat (Kirsch et al., 1952; Wolin et al., 1957; Ayres, 1960) indicate that Ps. geniculata was the most frequently encountered species. In other investigations (Marriot, Naumann, Stringer & Hendrick, 1967; Stringer et al., 1969) it was stated that Ps. fragi formed upwards of 50% of the flora of prepacked beef. With studies on chicken (Barnes & Thornley, 1966) and turkeys (Barnes & Impey, 1968) stored at 1^{o} pigmented pseudomonads were dominant. It may well be that the argument as to whether fluorescent types are more significant in spoilage than non-pigmented types is irrelevant. Since the generation times of both types are of the same order (Barnes & Impey, 1968; see also Fig. 15) the determining factor as to which group becomes dominant may depend simply on the relative initial proportions of each.

TABLE 8

PROPERTIES OF PSEUDOMONAS ISOLATED FROM BEEF

Morphology	rods, single or in pairs
Gram reaction	-
Motility	+
Flagella	polar
Glucose utilization	oxidative
Catalase	+
Oxidase	+
Reducing compounds from gluconate	+
Starch hydrolysis	-
Growth at 4^{o}	+
Growth at 41^{o}	-

+, positive; -, negative.

TABLE 9

DIFFERENTIATION OF SPECIES OF AEROBIC PSEUDOMONADS (STANIER ET AL., 1966)

	Fluorescent group				Pseudomallei group		Acidovorans group		Alcaligenes group					Isolates obtained in present study	
	Ps. aeruginosa	Ps. fluorescens	Ps. putida	Ps. multivorans	Ps. pseudomallei	Ps. mallei	Ps. acidovorans	Ps. testosteroni	Ps. alcaligenes	Ps. pseudoalcaligenes	Ps. stutzeri	Ps. maltophilia	Ps. lemoigneï	Pigmented	Non-pigmented
No. of flagella	1	>1	>1	>1	>1	0	>1	>1	1	1	1	>1	1	1 or >1	1 or >1
Poly-β-OH butyrate as cellular reserve	-	-	-	+	+	+	+	+	-	V	-	-	+	N.T.	N.T.
Pigments (Fluorescent	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-
Pigments (Phenazine	+	V	-	V	-	-	-	-	-	-	-	-	-	-	-
Methionine requirement	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Denitrification	+	V	-	-	+	V	-	-	-	-	+	-	-	-	-
Growth at 4°	-	+	V	-	-	-	-	-	-	-	-	-	-	+	+
Growth at 41°	+	-	-	V	+	+	-	-	+	+	V	-	-	-	-
Extracellular hydrolases gelatin	+	+	-	+	+	+	-	-	+	V	+	+	-	V	V
poly-OH-butyrate	-	-	-	-	+	+	-	V	-	-	-	-	+	N.T.	N.T.
starch	-	-	-	-	+	V	-	-	-	-	+	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Arginine dihydrolase	+	+	+	-	+	+	-	-	+	V	-	-	-	+	V
Cleavage mechanism for diphenols	ortho						meta		None		ortho	None		N.K.	

+, positive; -, negative; V, variable; N.T., not tested; N.K., not known.

TABLE 10

ORGANIC COMPOUNDS USED BY PSEUDOMONAS (ISOLATED FROM BEEF) AS SOLE CARBON AND ENERGY SOURCE

Carbohydrates and sugar derivatives

D-glucose (100)
gluconate (100)
2-ketogluconate (100)
D-ribose (90)
D-xylose (88)
D-fructose (83)
D-mannose (81)
L-arabinose (79)
mucate (78)
D-galactose (66)
D-arabinose (58)
trehalose (54)
sucrose (25)
inulin*

fatty acids

caprate (98)
caprylate (98)
acetate (90)
pelargonate (63)
valerate (32)
propionate (10)
butyrate (9)
isovalerate*
caproate*

dicarboxylic acids

succinate (100)
fumarate (100)
glutarate (100)

hydroxyacids

D-malate (100)
DL-lactate (100)
D-tartrate (16)
meso-tartrate (16)
DL-S-hydroxybutyrate*
DL-glycerate*

miscellaneous organic acids

citrate (100)
α-ketoglutarate (100)
pyruvate (100)
itaconate (44)
mesaconate (44)
aconitate*

polyalcohols and glycols

glycerol (100)
mannitol (87)
meso-inositol (63)
2,3 butyleneglycol (29)
propyleneglycol (11)
sorbitol (4)

alcohols

none

non-nitrogenous aromatic and other cyclic compounds

p-hydroxybenzoate (87)
benzoate (83)
quinate (80)

aliphatic amino acids

L-aspartate (100)
L-glutamate (100)
γ-aminobutyrate (100)
β-alanine (97)
DL-arginine (87)
D-α-alanine (84)
L-ornithine (84)
DL-serine (80)
L-valine (50)
DL-leucine*
iso-leucine*
glycine*
L-lysine*
DL-citrulline*

amino acids and related compounds containing a ring structure

L-histidine (100)
L-pyroline (100)
L-tyrosine (100)
L-phenylamine (98)

Amines

putrescine (100)
spermine (100)
ethanolamine (74)

miscellaneous nitrogenous compounds

creatine (95)
hippurate (88)
betaine (72)
sarcosine (25)

% Strains positive are shown in brackets
* Growth on these substrates was scanty and difficult to score.

TABLE 11

NUTRITIONAL CHARACTERS FOR THE DIFFERENTIATION OF SPECIES OF AEROBIC PSEUDOMONADS (STANIER ET AL., 1966)

Utilization as sole carbon and energy source of:	Fluorescent group				Pseudomallei group		Acidovorans group		Alcaligenes group					Isolates obtained in the present study
	Ps. aeruginosa	Ps. fluorescens	Ps. putida	Ps. multivorans	Ps. pseudomallei	Ps. mallei	Ps. acidovorans	Ps. testosteroni	Ps. alcaligenes	Ps. pseudoalcaligenes	Ps. stutzeri	Ps. maltophilia	Ps. lemoignei	
D-Fucose	-	-	-	+	+	+	-	-	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+	-	-	-	-	+	+	-	+
Trehalose	-	+	-	+	+	+	-	-	-	-	-	+	-	+ (54)*
Cellobiose	-	-	-	+	+	+	-	-	-	-	-	+	-	-
Maltose	-	-	-	-(+)	+	V	-	-	-	-	+	+	-	-
Starch	-	-	-	-	+	V	-	-	-	-	+	-	-	-
Inositol	-	+	-	+	+	+	V	-	-	-	-	-	-	+ (63)
Mannitol	+	+	-(+)	+	+	+	+	-	-	-	V	-	-	+ (87)
Geraniol	+	-	-	-	-	-	-	-(+)	-	-	-	-	-	-
2-Ketogluconate	+	+	+	+	+	V	-	-	-	-	-	-	-	+
Maleate	-	-(+)	-	-	-	-	+	-	-	-	-	-	-	-
Glycollate	-	-(+)	-(+)	V	-	-	+	+	-	-	+	-	-	-
DL-Lactate	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Pelargonate	+	+	+	+	+	V	-	-	+	+	+	-	-	+
Adipate	+	V	-	+	+	V	+	+	-	-	-(+)	-	-	-
m-hydroxybenzoate	-	-(+)	-(+)	+	-	-	+	+	-	-	-	-	-	N.T.
Testosterone	-	-(+)	-(+)	+	-	-	-	+	-	-	-	-	-	-
Acetamide	+	-	V	V	-	-	+	-	-	-	-	-	-	-
Arginine	+	+	+	+	+	+	-	-	+	+	-	-	-	+ (87)
Valine	+	+	+	V	+	V	-	-	-	-	+	-	-	+ (50)
Norleucine	-	-	-	-	-	-	+	+	-	-	-	-	-	-
D-Tryptophan	-	-	-	-	-	-	+	-	-	-	-	-	-	-
δ-Aminovalerate	+	V	+	+	+	V	+	-	+	+	-	-	-	N.T.
Betaine	+	+	+	+	+	+	-	-	-	+	-(+)	-	-	+ (72)
Putrescine	+	+	+	+	+	V	-	-	-	+	+	-	-	+

+, positive; -(+), usually negative, occasionally positive; -, negative; *, number positive; N.T., not tested.

TABLE 12

CHARACTERISTICS USED IN THE INTERNAL SUBDIVISION

OF FLUORESCENT PSEUDOMONAS

	Ps. fluorescens	Ps. putida
Gelatin hydrolysis	+	-
Growth on inositol as sole C and energy source	+	-
" trehalose "	+	-
" creatine "	-	+
" hippurate "	-	+
No. of strains	9	10

+, positive; -, negative.

MICROBACTERIUM THERMOSPHACTUM

The importance of M. thermosphactum in the spoilage of meats was discussed earlier; the following is concerned with its physiological and morphological properties and taxonomy.

The collection of strains studied was assembled from organisms recovered from beef used in the present experiments together with isolates from pork and lamb (received from Mr. A.W. Knight) and strains sent by Drs. A.G. Kitchell and G.A. Gardner. Type cultures of M. thermosphactum NCIB 10018, M. flavum NCIB 8707 and M. lacticum NCIB 8541 were also included in the study. All the organisms were Gram positive, nonsporeforming, non-motile rods. Results obtained in a detailed investigation of the morphological and physiological characteristics of these organisms are given below.

Morphology. When Gram-stained films of M. thermosphactum colonies were examined, it was noted that coccobacilli in relatively long chains predominated. However, in confirmation of earlier results (McLean & Sulzbacher, 1953) occasional long rods were also observed. This mixture of morphological types was found even after repeated subculturing to ensure purity. Pleomorphism in M. thermosphactum was also noted by David & Board (unpublished). In attempting to explain the occurrence of the variable morphology, these workers followed the growth of M. thermosphactum in agar slide cultures. It was shown that during incubation the coccal form grew into the

long rod form which eventually fragmented to give shorter rods and finally coccobacilli. Similar techniques were used in the present study, and in general the results agree with those obtained by David & Board. Plate 1 shows the development of a typical strain of M. thermosphactum growing on a Heart Infusion Agar slide incubated at c 20^{o}. It will be seen that the initial seeding consisted of small coccobacilli. After 3 h the coccobacilli had divided giving four daughter cells of similar morphology. These grew into rods (4 h and 5.5 h) which divided (6.5 h) producing daughter cells having rod form morphology. During subsequent incubation (7 h and 10 h) further multiplication took place, the cells remaining attached so that long kinked chains were formed (10 h). The long chains persisted, but after 24 h the cells had reverted to the coccobacillary form.

Shadowed preparations of cells of varying morphology were examined under the electron microscope. Plate 2 (a,b) shows the coccobacillary and long rod form respectively. In preparations of the long rods, an interesting feature was the occurrence of clumps of cells which were probably identical with the "large bodies" described by McLean & Sulzbacher (1953).

Electron micrographs of thin sections of short rods are shown in Plate 3. In the central regions of the cytoplasm nuclear material was evident which in Plate 3(a) is seen to be partitioning between each of the daughter cells. Septum formation and mode of division was typical of that for Gram positive organisms, i.e. a common cross wall was laid down and completed before

separation took place. With actively multiplying Gram negative bacteria, the cells are normally pinched off. Mesosomes associated with the cytoplasmic membrane and inclusions identified as polymetaphosphate granuals were also observed (Plate 3b).

Sections of the long rod form (Plate 4) confirmed that they were authentic filamentous rods (c five times the length of the coccobacilli) having no cross walls. Also in these preparations, large aggregates thought to consist of polyhydroxybutyrate were noted (Plate 4).

The cell wall and membrane were found to be typical for Gram positive organisms (Reynolds, 1968). Thus no evidence of layering in the wall was detected, whereas the membrane was found to be triple layered, i.e. an electron transparent region sandwiched between two electron dense layers (Plate 5). Dimensions of the wall and membrane were c 15 and 8 mμ respectively.

Freeze etched preparations are shown in Plates 6 and 7. Cell walls, membranes and cytoplasm can be seen. The inner surface of the cell wall appeared pitted (Plate 7). Similar structures were noted by Hurst & Stubbs (1969). These authors postulated that the pores were inverted truncated cones into which plugs could fit, and suggested that they might be involved in diffusion of the large molecules into the cell or in the absorption of phage.

Although it has been noted above that M. thermosphactum is Gram positive, Gram negative elements were occasionally observed especially as part of a chain of short rods. This has been

recorded by others (McLean & Sulzbacher, 1953; Weidemann, 1965; Gardner, 1966), and electron micrographs obtained in the present study offered a possible explanation for the variation. Thus in Plate 8 it can be seen that a pair of cells of comparitively low electron density were sandwiched between normal cells. The abnormal cells appeared to have weakened walls, these having been pushed in by growth of the normal cells. With these cells at a later stage, leakage of the cytoplasm might occur (Plate 9) and it is conceivable that they would give an apparent Gram negative reaction.

The morphological properties, in particular the growth cycle, discussed above were not found with M. lacticum and M. flavum. When grown under identical conditions, neither of these organisms showed the change from one morphological form to another. The variable morphology noted with M. thermosphactum is, however, a property of other groups of microorganisms e. g. Arthrobacter and Kurthia. With Arthrobacter Gram negative pleomorphic rods are present in young cultures, whereas irregular masses of Gram positive cocci are dominant by 1 - 3 d (Conn & Dimmick, 1948). More recent studies (Ensign & Wolf, 1964) have shown that the sphere-rod morphogenisis can be nutritionally controlled. Thus, a chemically defined medium was developed in which the growth of A.crystallopoietes was restricted to the coccoid form. Furthermore the rod form could be induced by the addition of single amino acids such as L-arginine, L. phenylalanine, L-asparagine, etc. to the medium. Other investigations (Krulwich, Ensign, Tipper & Strominger, 1967 a,b) revealed differences in the walls of coccoid

and rod forms of A. crystallopoietes. These workers found that peptidoglycan from coccoid cell walls contained polysaccharide of shorter chain length than that found in the rod form. Moreover, differences were also found in the peptide fractions, and cross linking bridges. It was suggested that these dissimilarities indicated that the sphere cell wall was probably a more loosely knit macromolecule than was the rod cell wall; thus providing a possible explanation for the occurrence of the two distinct forms.

Recent studies (Gardner, 1969) on the morphology of Kurthia zopfii showed that this organism possessed a growth cycle reminiscent in many ways to that found in M. thermosphactum (Plate 1). Thus when subcultured on to fresh medium, coccoid forms of K. zopfii developed into chains of rods then filaments, which eventually formed coils. Finally, breakage of the coils occurred, which would presumably give rise to the coccoid form.

At present it is not known whether or not the growth cycle found in M. thermosphactum is merely the result of the physical and chemical properties of the growth conditions. It would seem however a characteristic useful for routine identification.

Physiology. Table 13 gives some of the properties of M. thermosphactum, M. flavum and M. lacticum. It will be seen that growth temperatures, thermal resistance and cytochrome content

were the features which separated M. thermosphactum from the other two species. The psychrophilic nature of M. thermosphactum was also noted by Brownlie (1966) who reported that the growth occurred at 0^{o} but not at 35^{o}, and that the optimum was c 25^{o}.

In confirmation of the findings of others (Barlow & Kitchell, 1966) gas production from glucose fermentation was not detected. McLean & Sulzbacher (1953) reported that a small amount of CO_2 was formed. This may have arisen from acetoin production (Table 13) which involves condensation of 2 pyruvate molecules, and the liberation of CO_2. The main product of glucose fermentation was lactic acid which accounted for upwards of 75% of the glucose utilized. Other metabolic products detected were acetic and propionic acids (at levels equivalent to c 1% of glucose utilized) together with trace amounts of isobutyric, n-butyric, iso-valeric and n-valeric acids. It is interesting to note that no cis-aconitase activity was detected in extracts of M. thermosphactum, suggesting that this organism lacks a complete Tricarboxylic Acid Cycle. Further evidence supporting this view was obtained by Dr. R.H. Dainty (unpublished observations) who found that whole cells of M. thermosphactum were unable to utilize intermediates of the TCA cycle.

In a study of the cytochrome components of M. thermosphactum it was shown that growth conditions (incubation temperature and medium) influenced the formation of iron-prophyrin compounds. Table 14 summarizes the results obtained which were reported in detail elsewhere (Davidson & Hartree, 1968). It will be seen

that with cells grown at 20° in APT broth, cytochrome bands were clearly seen whereas at 30° none were detected. With cells grown in Heart Infusion Broth at 20° or 30° cytochrome bands were very weak. These results correlate well with the catalase and benzidine reactions. Thus at 30° M. thermosphactum almost invariably gave a negative reaction in both tests and, therefore, might well be confused with the lactic acid bacteria. It is for this reason that 20° incubation should be used when characterizing M. thermosphactum.

Taxonomy. The genus Microbacterium was proposed by Orla-Jensen (1919) to include heat resistant Gram positive catalase positive asporogenous rods isolated from milk and milk products.

Four species were recognized, Microbacterium lacticum, Microbacterium flavum, Microbacterium mesentericum and Microbacterium liquefaciens. In the 5th edition of Bergey's Manual (1939), Microbacterium was assigned to the family Bacteriaceae. The next edition (6th) (1948) transferred the genus to the tribe Lactobacilleae in the Lactobacteriaceae. M. liquefaciens was placed in the appendix, and M. mesentericum renamed Nocardia mesenterica. The current edition (7th) of the manual (1957) excluded M.liquefaciens, and Microbacterium (comprising M. flavum and M. lacticum) was transferred to Corynebacteriaceae.

The taxonomic position of Microbacterium still remains uncertain. It was proposed (Jensen, 1934; Abd-el-Malek & Gibson, 1952;

Jayne-Williams & Skerman, 1966) that the remaining species(M.flavum and M. lacticum) in the genus should be allocated to Corynebacterium. On the other hand, Speck (1943) stated that this would prove confusing and therefore recommended the retention of Microbacterium as a separate genus. Robinson (1966 a,b)supported this view in part, but proposed that on the basis of cell wall composition and other properties M. flavum be transferred to Corynebacterium, leaving the generic name Microbacterium to include M. lacticum and M. liquefaciens.

The exact systematic position of Microbacterium thermosphactum is also uncertain. Its similarity in some respects to the lactic acid bacteria has been stressed (Diebel & Evans, 1960; Barlow & Kitchell, 1966). In more recent studies, Davis, Fomin, Wilson & Newton (1969) used numerical taxonomy techniques in an attempt to classify Listeria and related organisms which included strains of M. thermosphactum. They concluded that M. thermosphactum together with Listeria and Erysipelothrix showed closer phenotypical relations to the Lactobacillaceae than to certain members of the Corynebacteriaceae. However, in these studies only one lactobacillus was examined, which would appear insufficient. In addition, Davis and his co-workers reported that, contrary to the results obtained here (Table 13), M. thermosphactum did not produce acetoin from glucose and failed to give a strong positive catalase reaction.

The work reported here suggests that M. thermosphactum should not be transferred to the Lactobacillaceae. This conclusion is based mainly on the findings that this organism possesses a

functional cytochrome system, is catalase positive and has morphological characteristics more akin to the Corynebacteriaceae. Of interest too, is the recent finding (Shaw & Stead, 1970) that M. thermosphactum did not possess a galactosylglucosyl diglyceride, a glycolipid which has been found to occur in all the lactic acid bacteria so far investigated (Shaw & Baddiley, 1968)

With regard to cytochrome and catalase, it should be borne in mind that some lactic acid bacteria can form catalase and functional cytochrome when supplied with haematin. Apparently these organisms produce the apoenzyme, but are unable to synthesize the iron porphyrin part of the molecule. Recently Bryan-Jones & Whittenbury (1969) have shown that Streptococcus faecalis formed a b_2-type cytochrome when grown on medium supplemented with haematin at a final concentration of 50 ug/ml. They concluded that S. faecalis and possibly other lactic acid bacteria, could be considered metabolic "cripples", lacking a Krebs cycle but forming iron porphyrin enzymes when supplied with haematin. It is interesting to speculate that M. thermosphactum through its ability to synthesize complete cytochromes and catalase, but still lacking a Krebs cycle, might be considered only one step removed from organisms such as S. faecalis.

Although in our opinion M. thermosphactum should be retained in the Corynebacteriaceae at present, it is debatable whether Microbacterium is the most suitable genus since differences between M. thermosphactum and the other two species were noted (Table 13). Moreover Davis & Newton (1969) in a study of similarity values, found that M. lacticum and M. thermosphactum were not linked even

at the 60% level. Investigations on the lipid composition of M. thermosphactum (Shaw & Stead, 1970) also revealed significant differences between this organism and M. lacticum.

TABLE 13

PROPERTIES OF MICROBACTERIA

Property	Response of M. thermosphactum	M. lacticum	M. flavum
Action on glucose	fermentative	fermentative	fermentative
VP test	+	+	-
Methyl red test	+	+	+
Reduction of nitrate	-	-	-
Production of catalase	+	+	+
Benzidine reaction	+	+	+
Cytochromes detected	a, b_1, a_3	a, b, c	a, b, c
Growth at 4^o	+	-	-
Growth at 20^o	+	+	+
Growth at 30^o	+	+	+
Growth at 37^o	-	+	+
Decimal reduction times	D_{50} = 2.5 min	D_{70} = 4 min	D_{65} = 2 min

TABLE 14

CYTOCHROME AND NADH OXIDASE IN M. THERMOSPHACTUM AND IN THE OTHER TWO SPECIES OF THE GENUS MICROBACTERIUM

	Strain	Medium	Cytochrome components in cells grown at 20°	Cytochrome components in cells grown at 30°	Percentage inhibition by 3 mM-HCN of NADH oxidase from cells grown at 20°	Percentage inhibition by 3 mM-HCN of NADH oxidase from cells grown at 30°
M. thermosphactum	NCIB 10018	APT	$a > b_1$		About 90	<5
	G202	APT	$a < b_1$	Bands very weak or not detected	About 90	
	WR7	APT	$a < b_1$		About 90	
	H43	APT	$a < b_1$		About 90	
	NCIB 10018	Heart infusion	Very weak bands of a and b_1			
	H43	Heart infusion				
M. lacticum	NCIB 8541	APT		a, b, c		
M. flavum	NCIB 8707	APT		a, b, c		

Strains G202 and WR7 were obtained from Drs.G.Gardner & A.Kitchell respectively. Strain H43 was typical of those isolated in the present study.

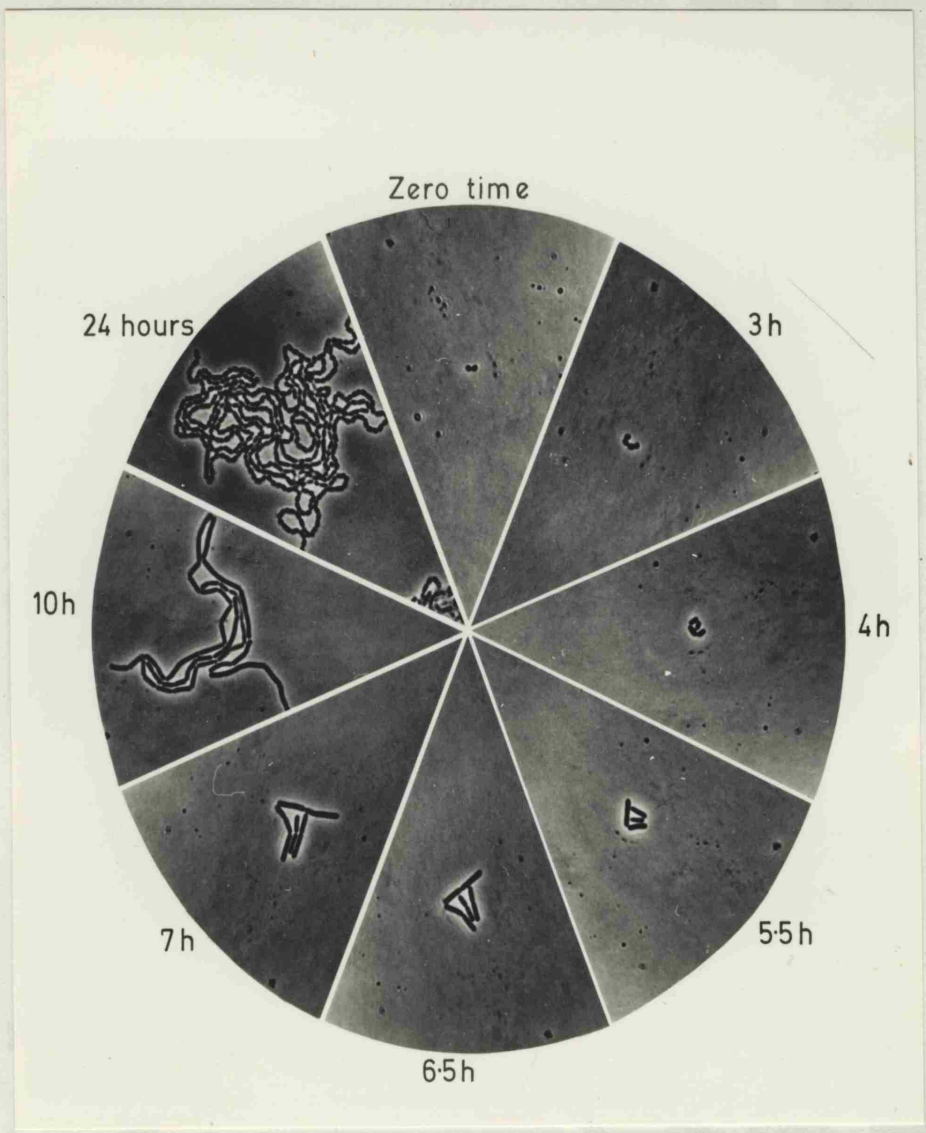
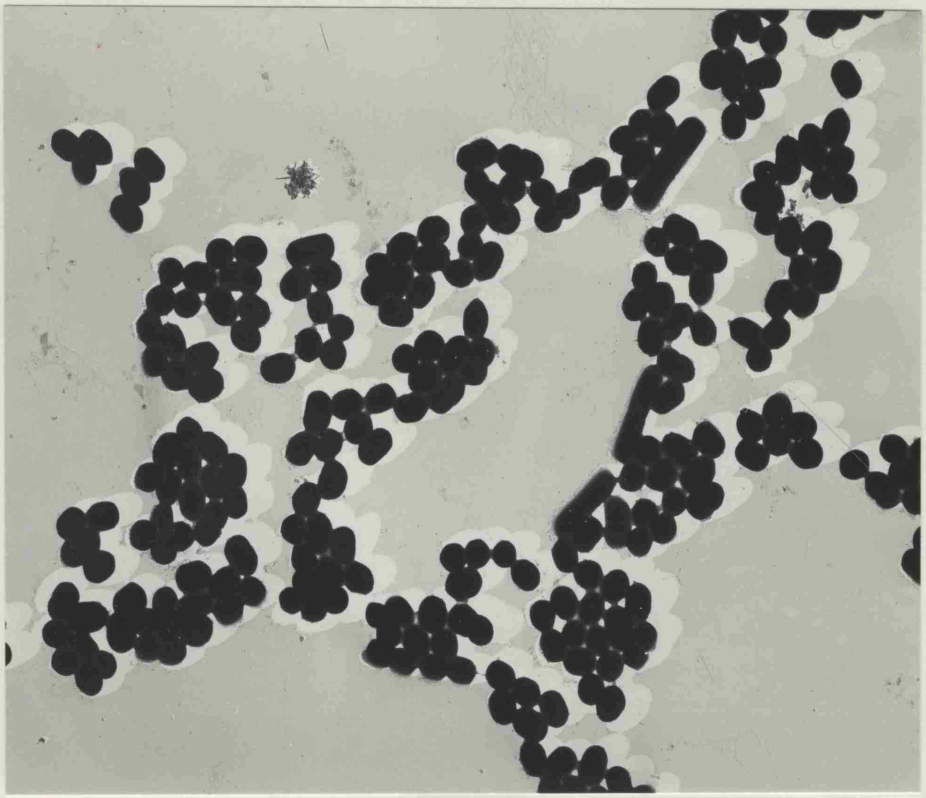
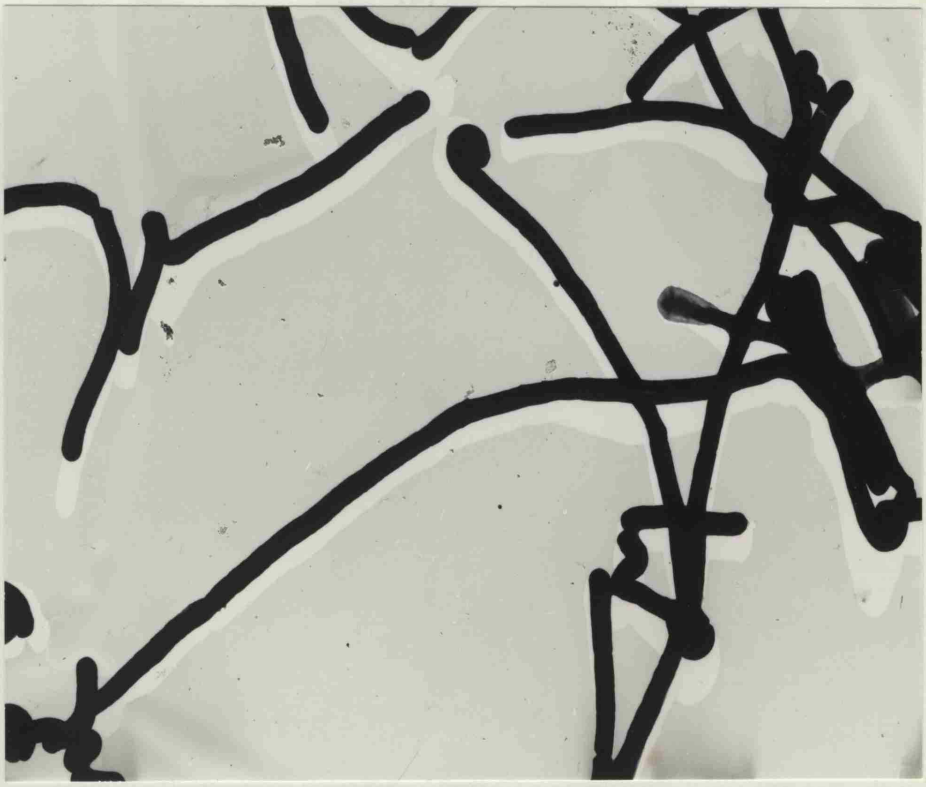


PLATE 1. Development of M. thermosphactum on Heart Infusion Agar(x625).

Abbreviations used in Plates 3–9: NM, nuclear material; R, ribosome; PMP, polymetaphosphate; M, mesosome; PHB, polyhydroxybutyrate; CW, cell wall; CM, cytoplasmic membrane; S, cross wall; I.S., inner surface; O.S., outer surface.



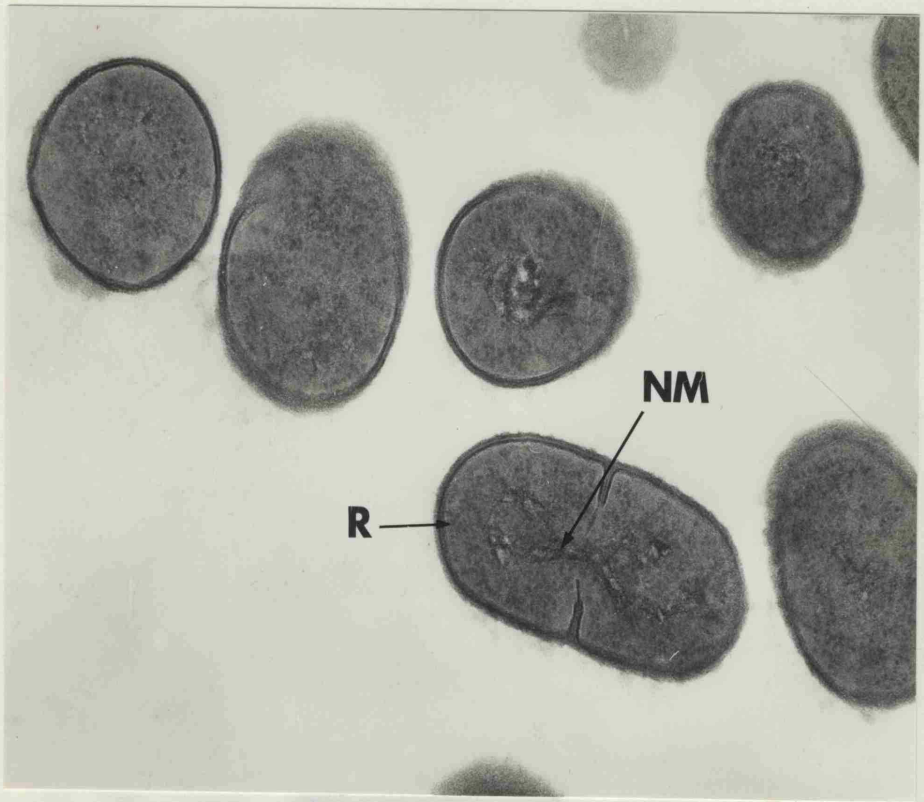
(a)



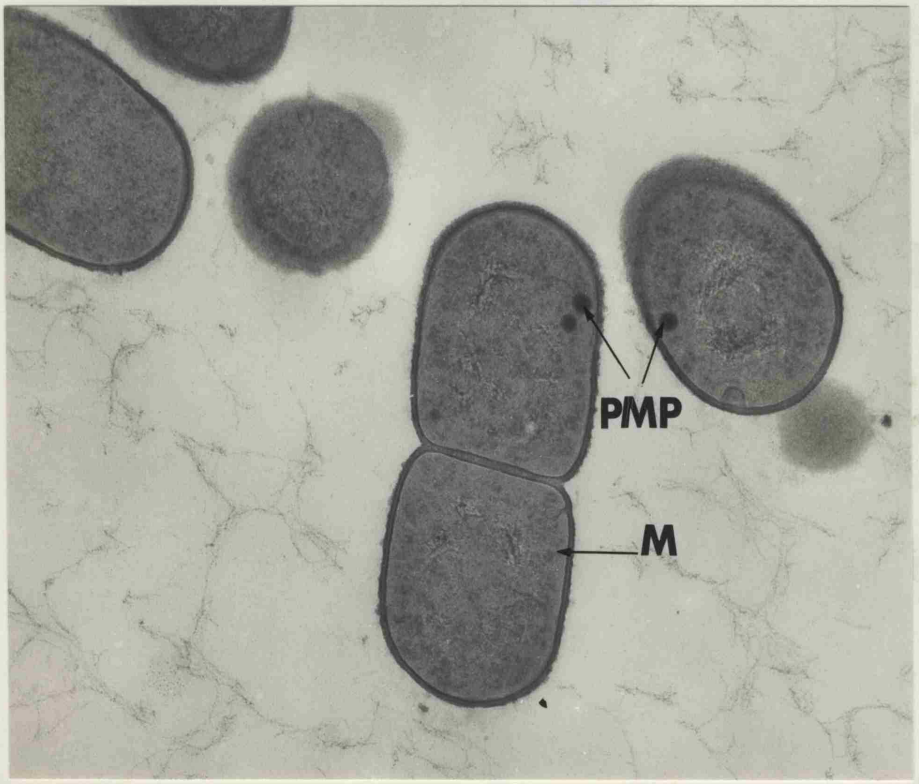
(b)

PLATE 2. (a) Shadowed preparation of coccobacillary form of M. thermosphactum (x 6000)

(b) Shadowed preparation of long rod form of M. thermosphactum (x 6000)



(a)



(b)

PLATE 3. (a) Section of coccobacillary form of M.thermosphactum. Cross walls in early stage are evident (x 50,000).

(b) Section of coccobacillary form of M. thermosphactum. Polymetaphosphate inclusions and a mesosome associated with the membrane are evident (x50,000).

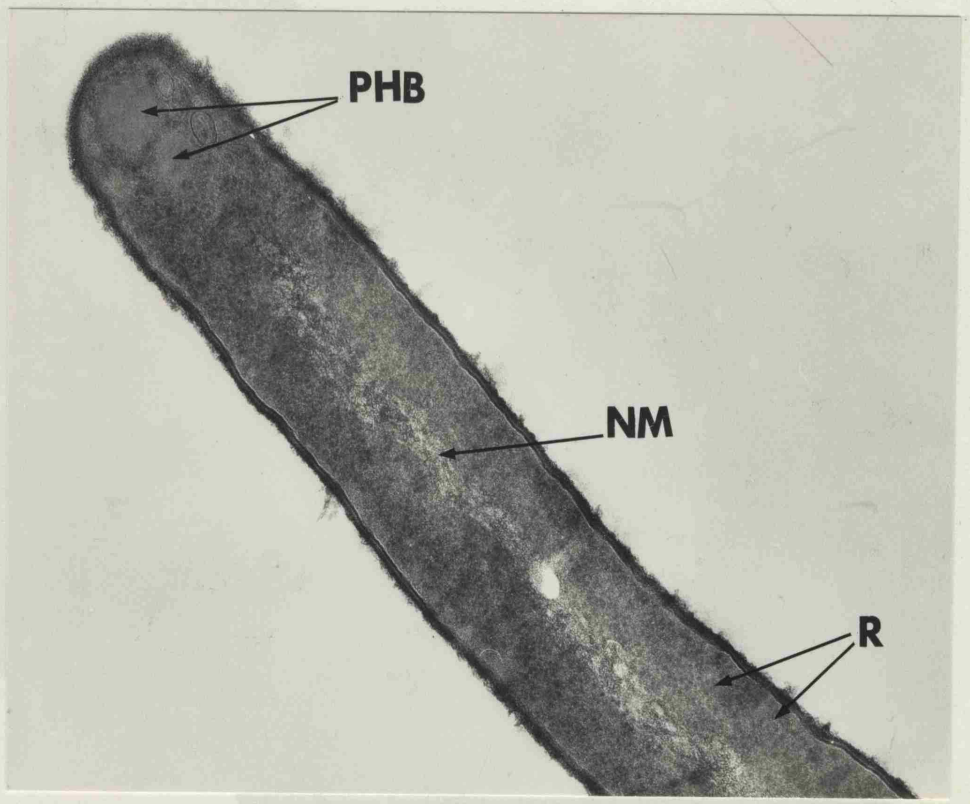


PLATE 4. Section of long rod form of M. thermosphactum. polyhydroxybutyrate inclusions are evident (x 50,000).

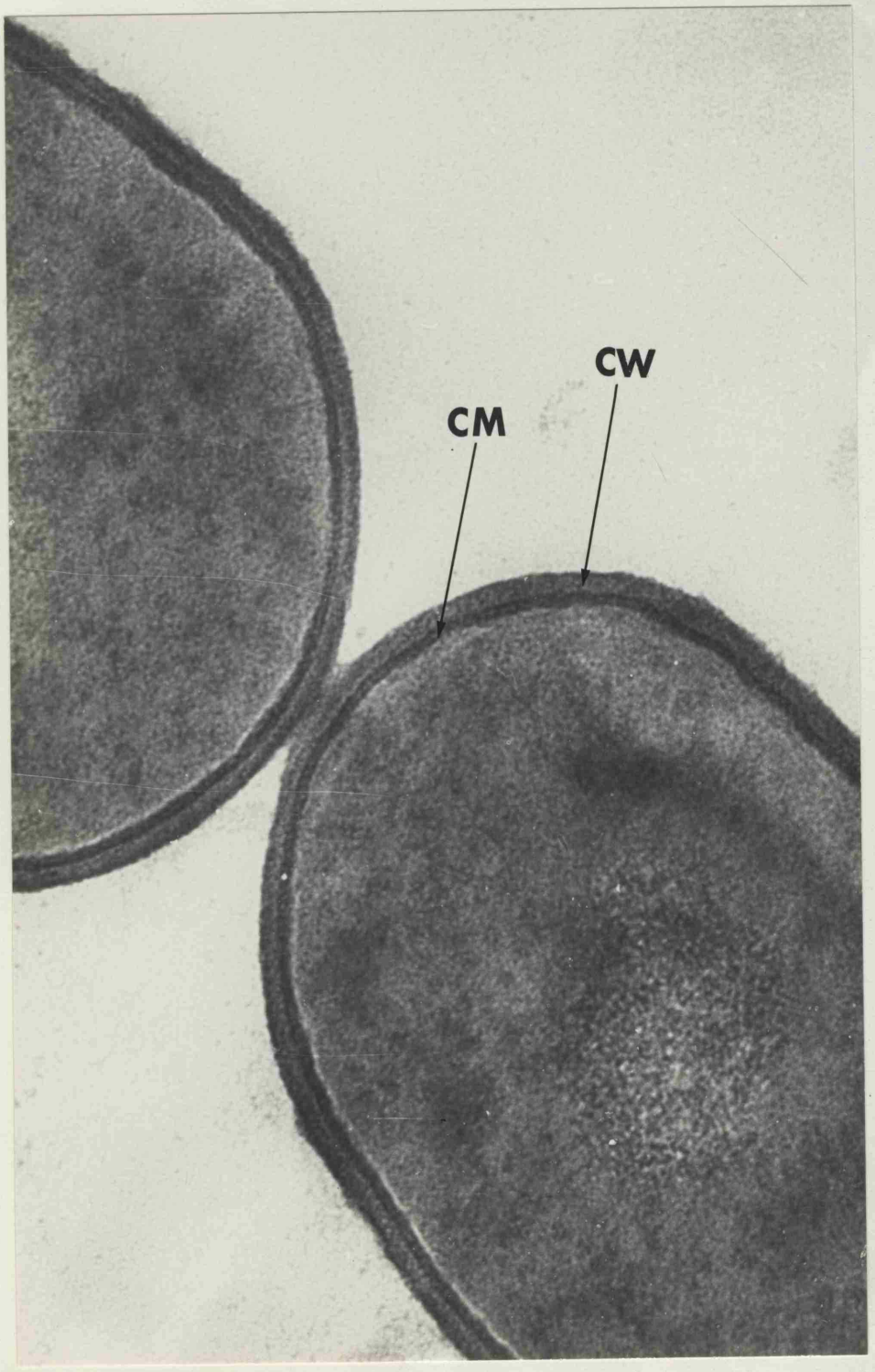


PLATE 5. Section of M. thermosphactum showing organization of cytoplasmic membrane (x 187,500).

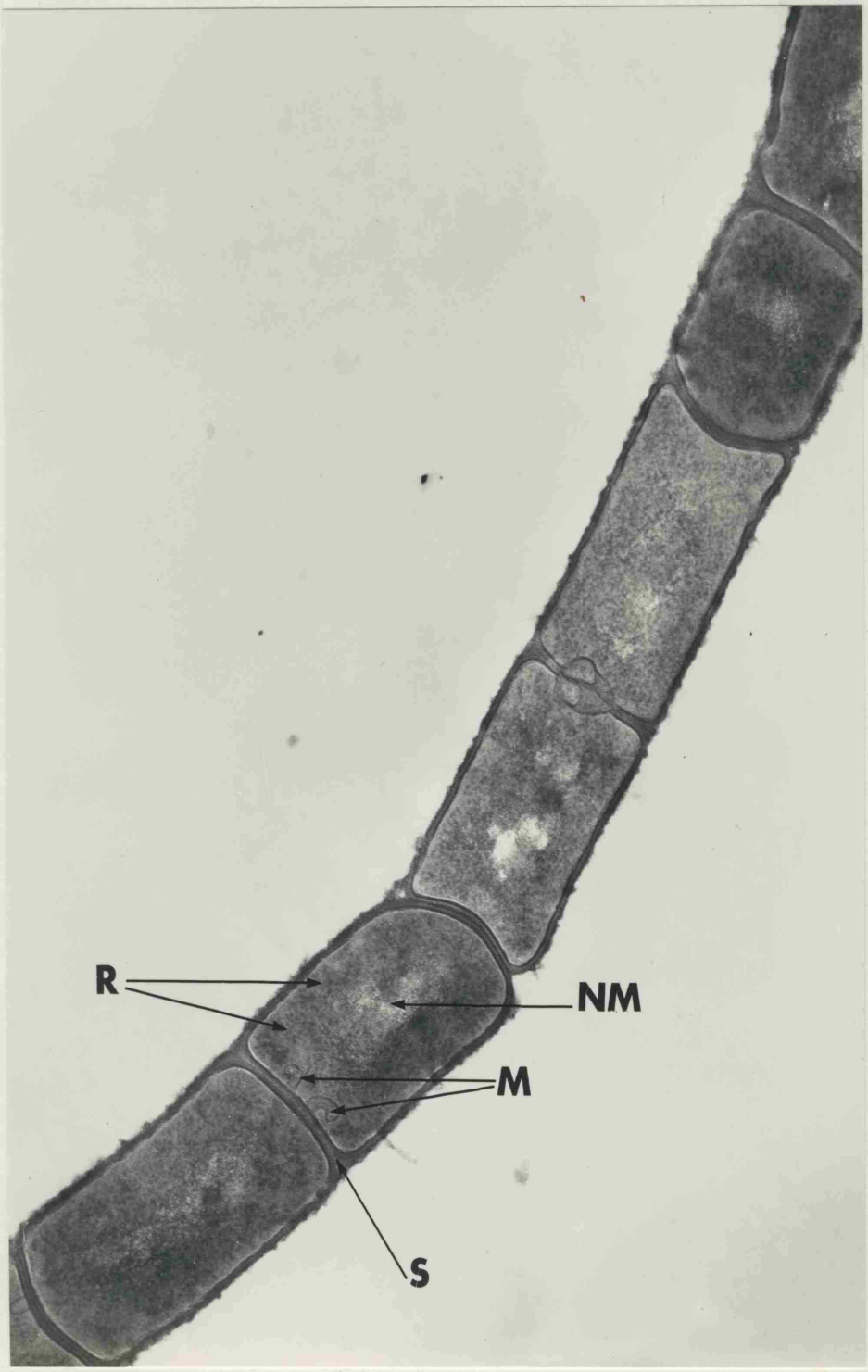


PLATE 6. Section of chain of coccobacillary form of M.thermosphactum. Mesosomes and cross walls are evident (x 50,000).

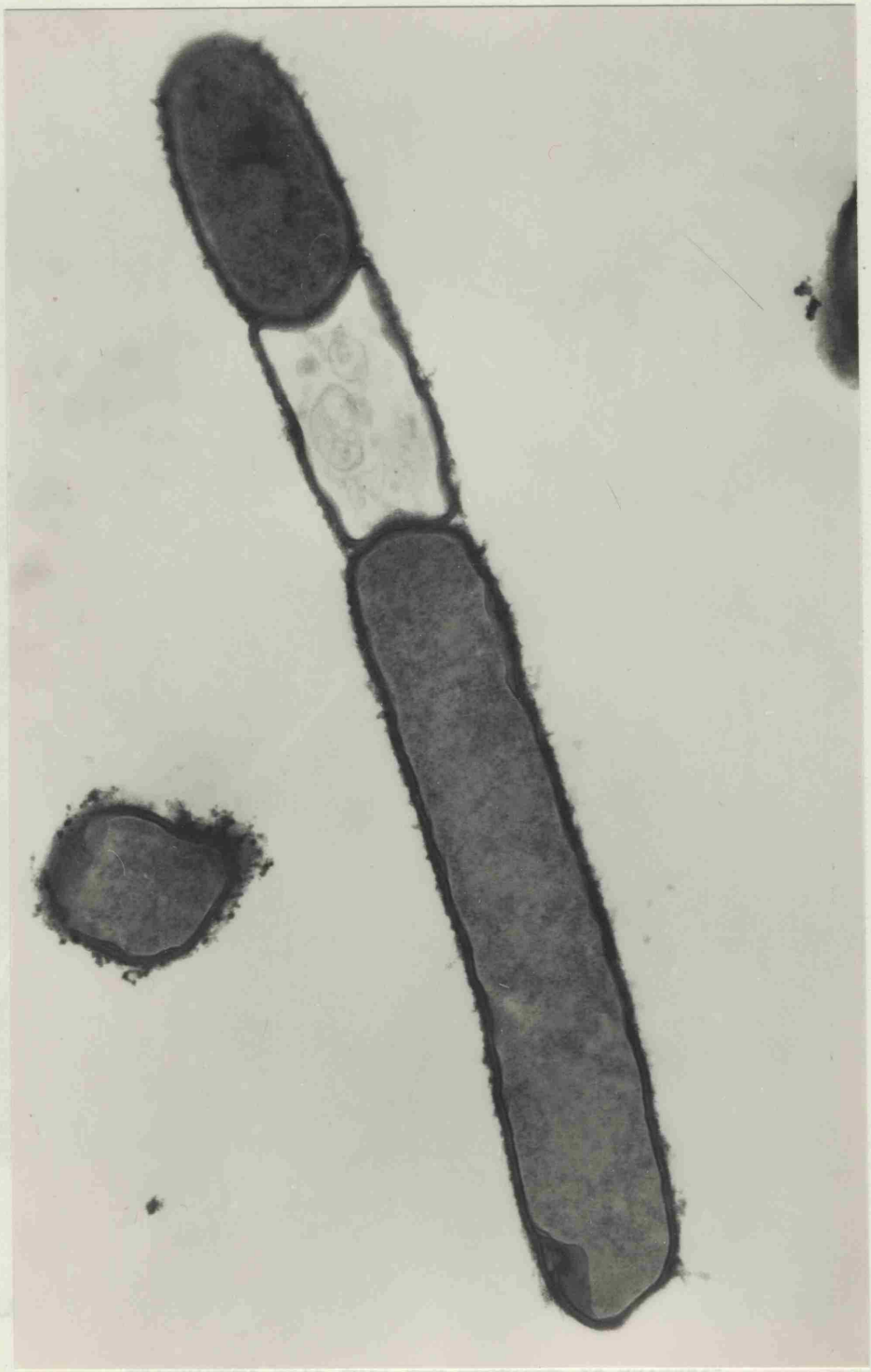


PLATE 7. Section of chain of coccobacillary form of M. thermosphactum, showing electron transparent cell sandwiched between normal cells (x 50,000).

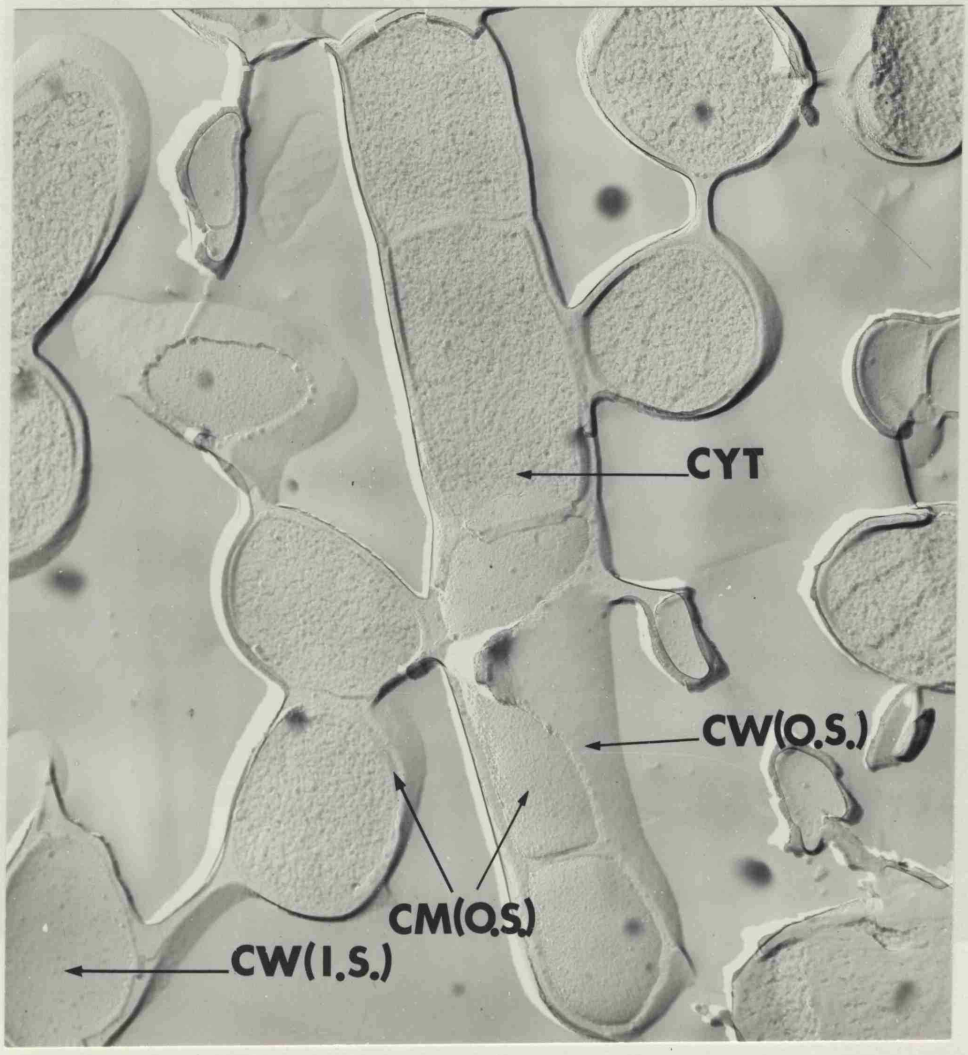


PLATE 8. Freeze etched preparation of M. thermosphactum, showing cytoplasm, cell wall and membrane (x 50,000).

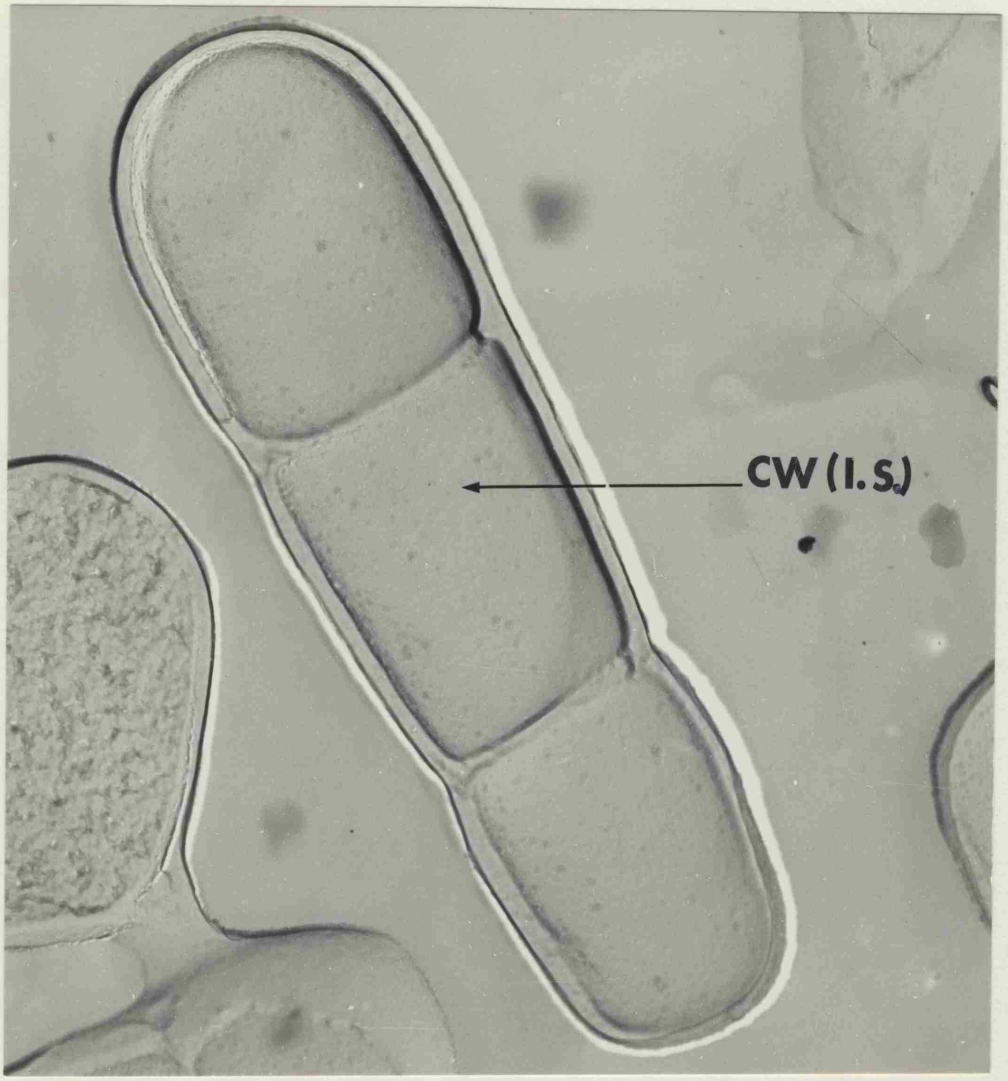


PLATE 9. Freeze etched preparation of M. thermosphactum showing structure of inner surface of the cell wall (x 100,000).

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Some Morphological and Physiological Properties of *Microbacterium thermosphactum*

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SUMMARY. The properties of strains of *Microbacterium thermosphactum* obtained from various sources were investigated. All exhibited pleomorphism and occurred both as coccobacilli and as long rods. The change from one morphological form to another was followed by means of slide culture techniques. Electron micrographs of thin sections of *M. thermosphactum* were also examined. The medium and incubation temperature influenced the catalase and benzidine reactions. The practical implications and the possible taxonomic significance of the results are discussed.

THE NAME *Microbacterium thermosphactum* was proposed (McLean & Sulzbacher, 1953) for a Gram positive, catalase positive, nonsporing fermentative organism that had been isolated from American pork sausages. This same organism probably constitutes the majority of the Gram positive rods which form a significant part of the microflora of British pork sausages (Dyett & Shelley, 1962); it outgrows the *Pseudomonas-Achromobacter* (*Acinetobacter*) group of organisms as the product sours during storage (Dowdell & Board, 1967). In the older literature reviewed by Haines (1937) there are several references to Gram positive rods being present on uncomminuted meat. In no instance do the characteristics of any of these organisms justify their identification with *M. thermosphactum*. In the past decade or so, however, organisms which could now be tentatively identified with *M. thermosphactum* have been recovered from beef (Rogers & McCleskey, 1957; Wolin, Evans & Niven, 1957; Ayres, 1960; Weidemann, 1965), poultry (Thornley, 1957), lamb (Barlow & Kitchell, 1966) and pork (Gardner, Carson & Patton, 1967). A common feature of most of these investigations is the use of storage conditions or wrapping materials which impede free gaseous exchange, and, according to Gardner *et al.* (1967), a depletion of oxygen together with an increase in the concentration of CO_2 selectively favours the growth of *M. thermosphactum*.

David & Board (unpublished) noted pleomorphism in pure cultures of *M. thermosphactum* growing on nutrient or plate count agar. For example, whilst organisms in the centre of the colony occurred as coccobacilli in short chains or irregular clusters those at the periphery occurred as long rods arranged in long chains. In slide culture the coccal form grew into the long rod form which then divided to give shorter rods and finally coccobacilli, a sequence which resembles superficially that occurring with *Arthrobacter* spp. (Conn & Dimmick, 1948; Stevenson, 1961). These observations were confirmed by one of us (C.M.D.), and it was noted that this cycle of events was influenced by the choice of medium. In addition to the potentially confusing pleomorphism, variable results were occasionally obtained with two tests, namely, the

catalase and benzidine tests. Although *M. thermosphactum* has been characterized adequately (McLean & Sulzbacher, 1953; Barlow & Kitchell, 1966; Gardner, 1966), these features might prove troublesome in routine bacteriological examination of meat and meat products and lead to possible confusion of *M. thermosphactum* with other coryneform bacteria and perhaps some lactic acid bacteria. It was for this reason that the present work was undertaken.

Materials and Methods

Physiological properties

Cultures (18 h) on Heart Infusion Agar (Difco) (HIA) were used as the inoculum for all tests. Incubation was at 20° unless otherwise stated.

Glucose utilization

The medium and methods proposed by the International Sub-committee on Staphylococci and Micrococci (1965) were used. Inoculated tubes were examined repeatedly during a period of 1 week. An acid reaction in the depth of the medium was taken to be evidence of anaerobic utilization of glucose.

Acetoin production and MR test

Organisms were grown in a medium containing (% w/v); peptone (Evans), 0·5; K_2HPO_4, 0·5; glucose, 0·5; pH, 7·0. After 5 days the culture was examined for acetoin production by Barritt's (1936) method and the pH was tested with methyl red.

Gas from glucose

Inoculated tubes containing the medium described by Gibson & Abd-El-Malek (1945) were sealed and examined for gas production during 2 weeks' incubation.

Fermentation products

4 oz medical flats were filled to the neck with the medium of McLean & Sulzbacher (1953). The sterile medium was steamed for *c.* 20 min, cooled to room temperature and then inoculated immediately. After 4 weeks at 20°, the medium was centrifuged and the supernatant examined. The Munson-Walker technique (Triebold & Aurand, 1963) and Barker & Summerson's (1941) method were used to estimate residual glucose and lactic acid, respectively. In addition, control sterile medium and the supernatant liquid from the cultures were analysed on a Pye Model 104 gas chromatograph fitted with flame ionization detectors (W. G. Pye & Co., Cambridge). Mixtures were separated in a nitrogen flow of 90 ml/min at 110° on a column (4 ft × 5 mm ID) containing Tween 80 (15% w/w) on Chromosorb W (60–80 mesh, Johns-Manville Co. Ltd., London) pretreated with phosphoric acid.

Nitrate reduction

Cultures in a medium containing (% w/v); peptone (Evans), 1·0; Na_2HPO_4, 0·5; Lab-Lemco, 0·3; yeast extract, 0·1; glucose, 0·1; $NaNO_3$, 0·02; pH, 7·2, were tested

after 5 days for the presence of nitrite, and if negative, for nitrate (Cowan & Steel, 1965).

Catalase production

H_2O_2 (5% w/v) was added to colonies on HIA or the APT medium of Evans & Niven (1951). Evolution of gas was taken as positive evidence of catalase.

Benzidine test (Diebel & Evans, 1960)

Benzidine dihydrochloride solution followed by H_2O_2 (5% w/v) was added to colonies on HIA or APT agar after incubation at 20 or 30°. The rapid development of a blue colour was taken as evidence of the presence of cytochrome.

Growth temperatures

Heart Infusion (HI) or APT broth cultures were incubated in a thermostatically controlled water bath at ($\pm$0·5°) 4, 20, 30 or 37°.

Thermal resistance

A known volume (0·2 ml) of 18 h HI broth cultures was added to 20 ml of a sterile 10% w/v skim-milk (Oxoid) solution. The skim-milk suspension was then dispensed in 2 ml amounts in 12 × 75 mm test tubes, ensuring that none came into contact with the upper walls of the tube. The inoculated tubes, 8 for each organism, were plugged with cotton wool and held in a thermostatically controlled water bath. At suitable time intervals, depending on the organism, 4 tubes were rapidly transferred to iced water, and the number of organisms surviving determined by surface plating techniques on Milk Agar (Oxoid) medium.

Morphology

Agar slide cultures

A phase contrast microscope (Zeiss photomicroscope) was used to follow the growth of isolated cells on the surface of either APT, HIA or plate count agar. Drops of medium were allowed to set on sterile slides, inoculated and sealed with a coverslip. To prevent evaporation the inoculated slides were placed in a glass chamber, securely clamped to the microscope stage. Photographs were taken on 35 mm Ilford FP3 film.

Electron microscopy

Cells were harvested by centrifuging Heart Infusion broth cultures, or from suspensions prepared from agar cultures. These were twice washed with 0·03 M Sørensens phosphate buffer (pH 7·2) and then fixed with glutaraldehyde and osmium tetroxide by the method described by Hamilton & Stubbs (1967). The fixed cells were washed in 3 changes of distilled water. The cells were recovered by centrifuging and resuspended in a small quantity of liquefied agar (at *c.* 50°). This was then cut into small pieces (0·5 mm^3) and dehydrated by passage through increasing concentrations of ethanol, and finally embedded in Araldite (Glauert & Glauert, 1958).

An LKB Ultratome III microtome (LKB, Stockholm) was used to cut thin sections of *c.* 700 Å thickness. These were stained with 1% (w/v) uranyl acetate in water and lead citrate (Reynolds, 1963), and examined in a JEM 7A electron microscope (Delvijem, Finchley, London). Fixed cells on carbon coated electron microscope specimen grids were shadowed at a nominal angle of 45° with a gold/palladium alloy. These were examined in a JEM 6A electron microscope fitted with an ACW low magnification device.

Results

The collection of *M. thermosphactum* was assembled from isolates recovered from beef and from strains received from Drs. Kitchell and Gardner, as well as type cultures of *M. thermosphactum* NCIB 10018, *M. flavum* NCIB 8707 and *M. lacticum* NCIB 8541. These organisms were all Gram positive, nonsporeforming, nonmotile rods.

Morphology

When HIA was used to recover organisms from stored beef, it was noted that some of the colonies (subsequently identified with *M. thermosphactum*—Table 1) consisted mainly of Gram positive coccobacilli in relatively long chains; a few long rods were also present. This situation was observed, even after repeated subculturing to ensure purity, and led to a detailed study of the morphology of the organism: the results reproduced in Plate 1 show those obtained with all the strains of *M. thermosphactum* examined. The initial seeding examined with the light microscope consisted of coccobacilli. This was confirmed when cells of the same age as the inoculum were examined by electron microscopy (Plate 2(*a*)). Within 3 h at *c.* 20° the coccobacilli divided (Plate 1) and the daughter cells grew into rods (5·5 h). The rods increased in length (6·5 h) and when division occurred, the cells remained associated, thus forming

TABLE 1

Properties of microbacteria

Characteristic	Response of		
	M. thermosphactum	*M. lacticum*	*M. flavum*
Action on glucose	fermentative	fermentative	fermentative
VP test	+	+	−
Methyl red test	+	+	+
Reduction of nitrate	−	−	−
Production of catalase	+	+	+
Benzidine reaction	+	+	+
Cytochromes detected*	*a*, b_1, a_3	*a*, *b*, *c*	*a*, *b*, *c*
Growth at 4°	+	−	−
Growth at 20°	+	+	+
Growth at 30°	+	+	+
Growth at 37°	−	+	+
Decimal reduction times†	D_{50}=2·5 min	D_{70} = 4 min	D_{65}= 2min

* Davidson & Hartree (1968).

† Calculated according to the methods of Katzin, Sandholzer & Strong (1943).

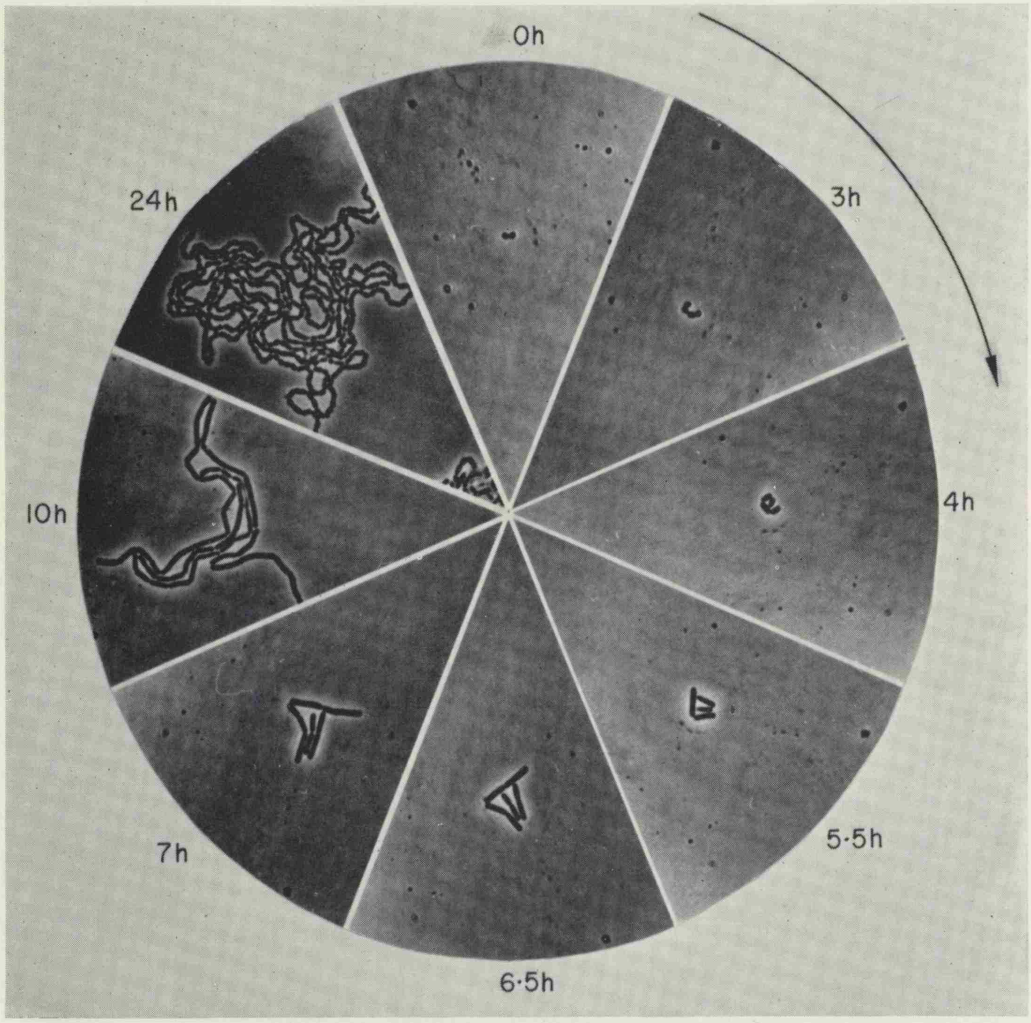

PLATE 1. Development of *M. thermosphactum* on Heart Infusion Agar (× 625).

Abbreviations used in Plates 3–6: NM, nuclear material; R, ribosome; PMP, polymetaphosphate; M, mesosome; PHB, polyhydroxybutyrate; CW, cell wall; CM, cytoplasmic membrane; S, cross wall.

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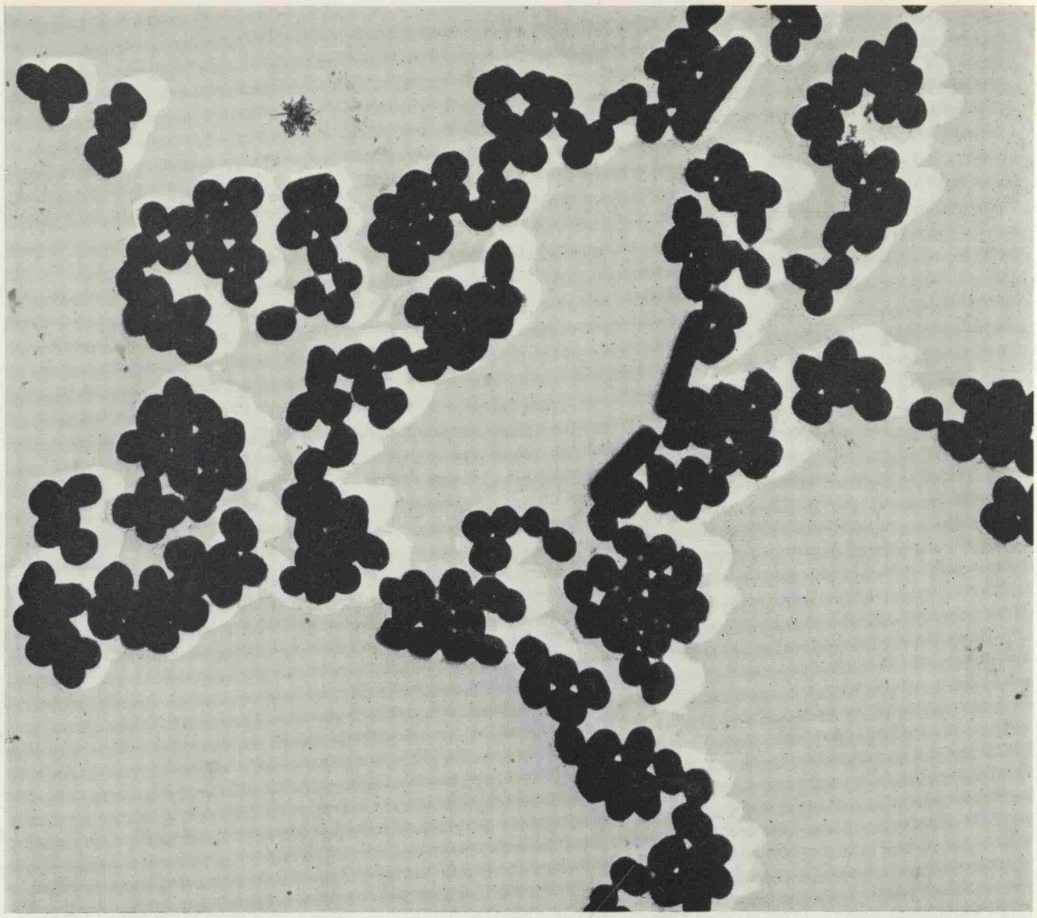
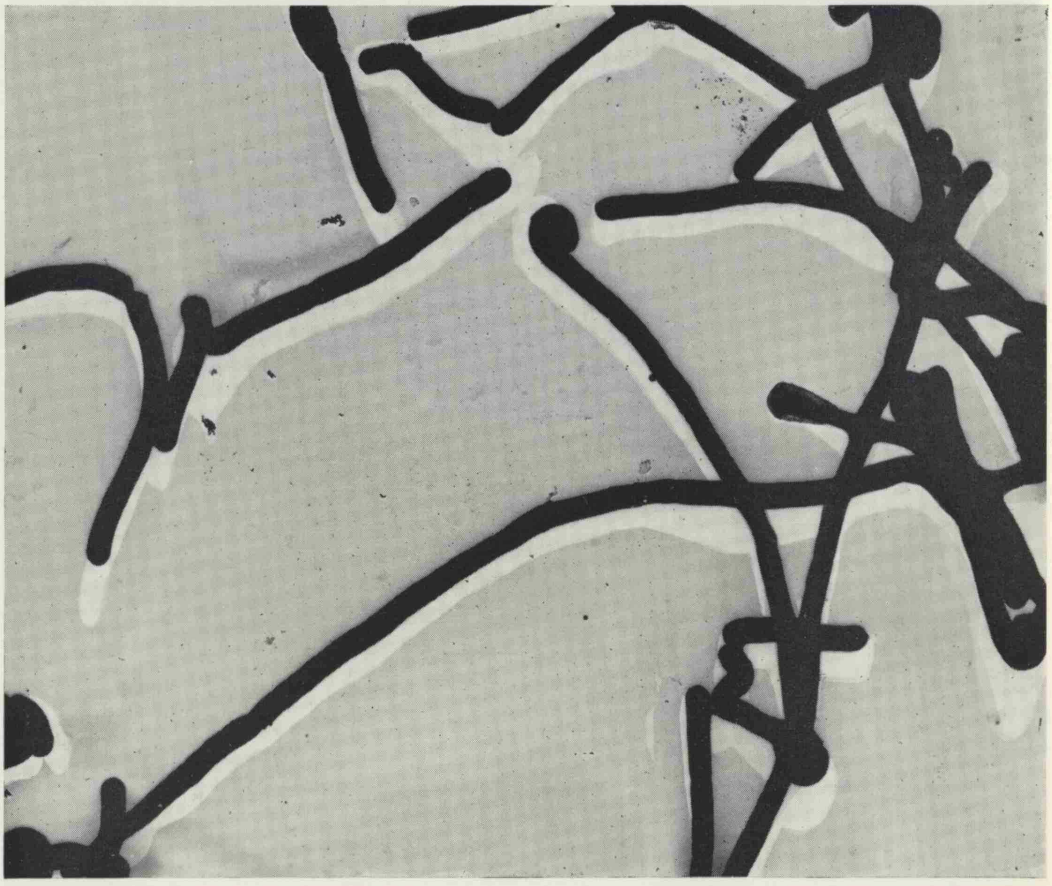
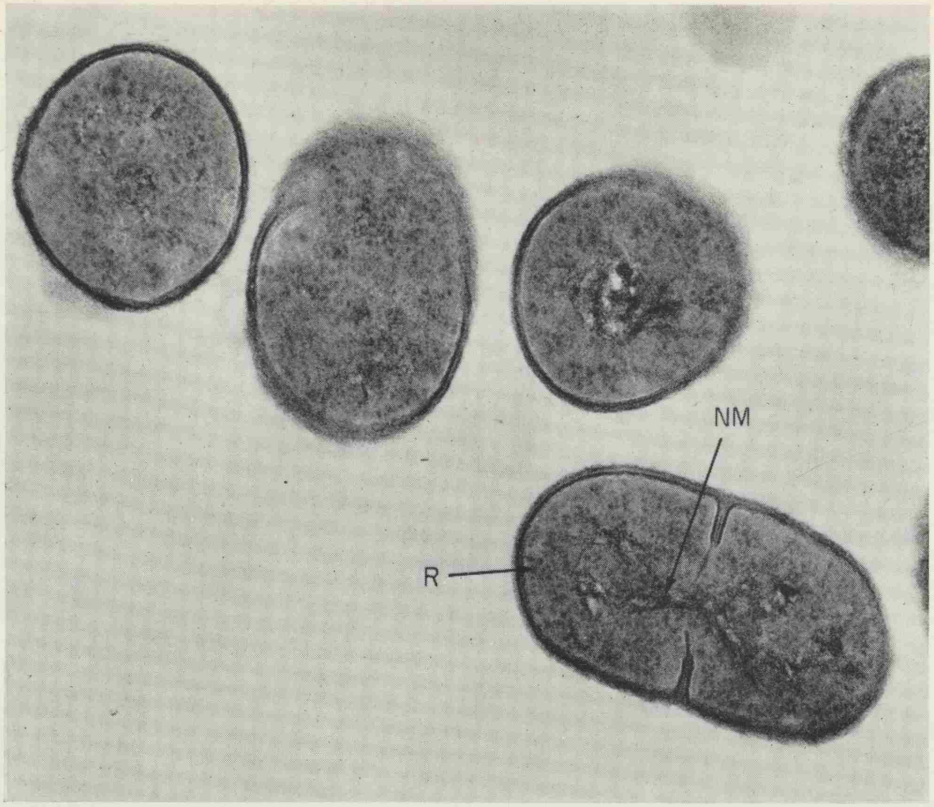


(*a*)

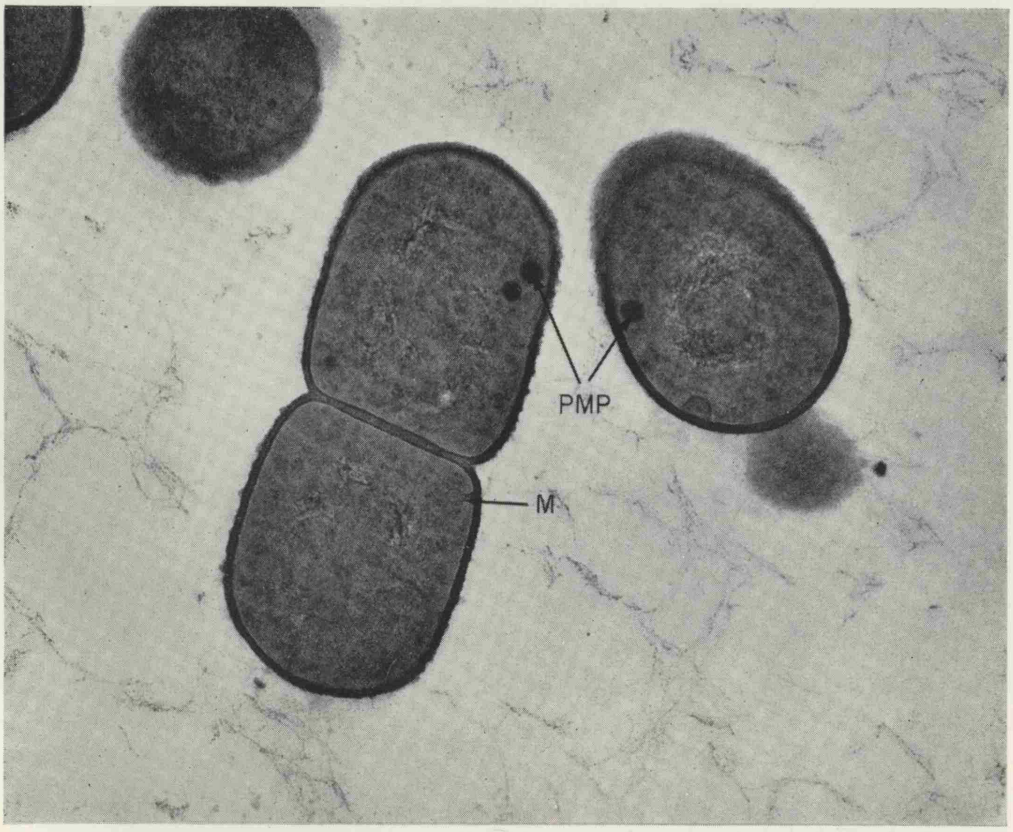


(*b*)

PLATE 2. (*a*) Shadowed preparation of coccobacillary form of *M. thermosphactum* (× 6000).
(*b*) Shadowed preparation of long rod form of *M. thermosphactum* (× 6000).



(*a*)



(*b*)

PLATE 3. (*a*) Section of coccobacillary form of *M. thermosphactum*. Cross walls in early stage are evident (× 50,000).

(*b*) Section of coccobacillary form of *M. thermosphactum*. Polymetaphosphate inclusions and a mesosome associated with the membrane are evident (× 50,000).

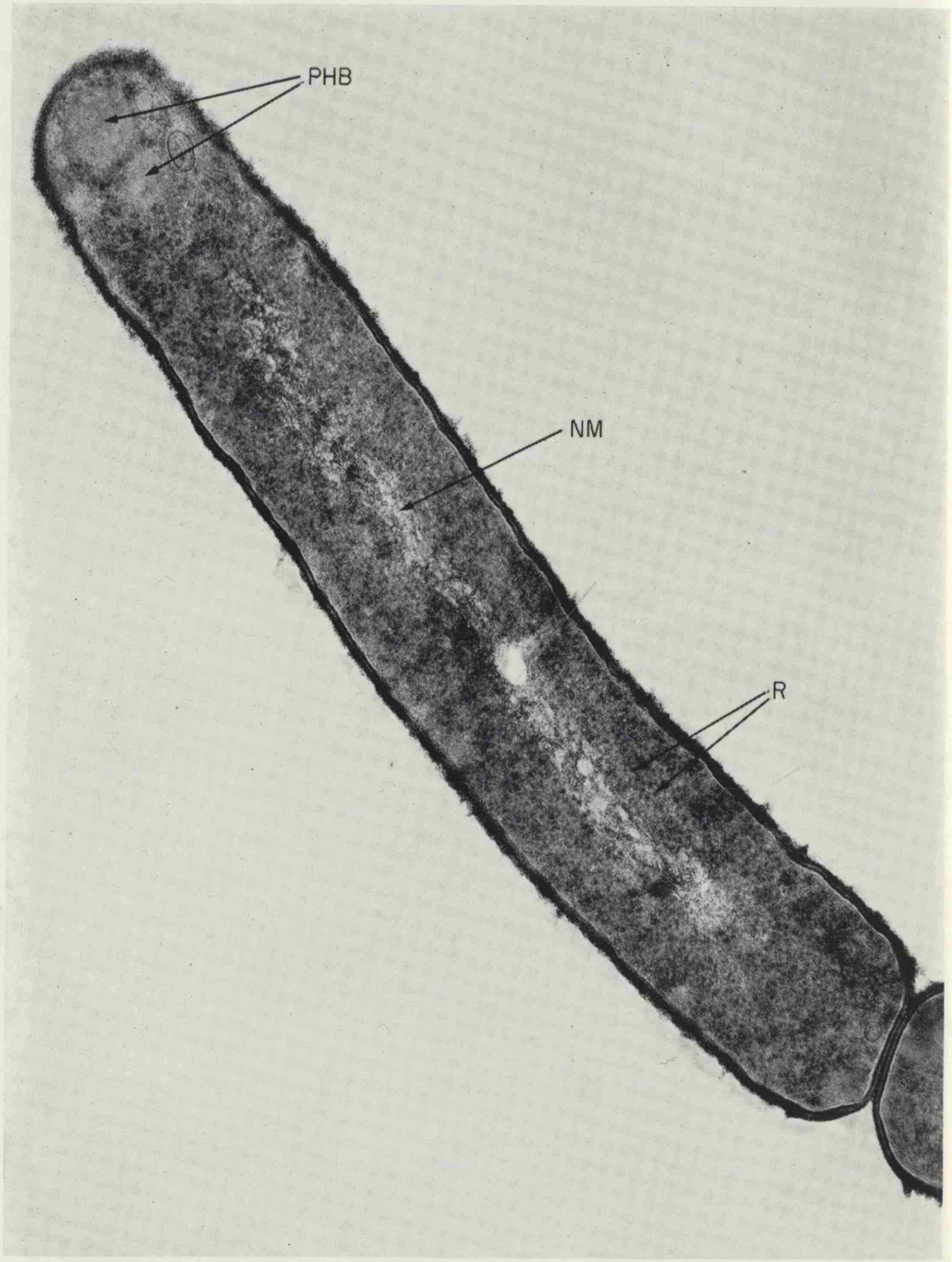


PLATE 4. Section of long rod form of *M. thermosphactum*. Polyhydroxybutyrate inclusions are evident (× 50,000).

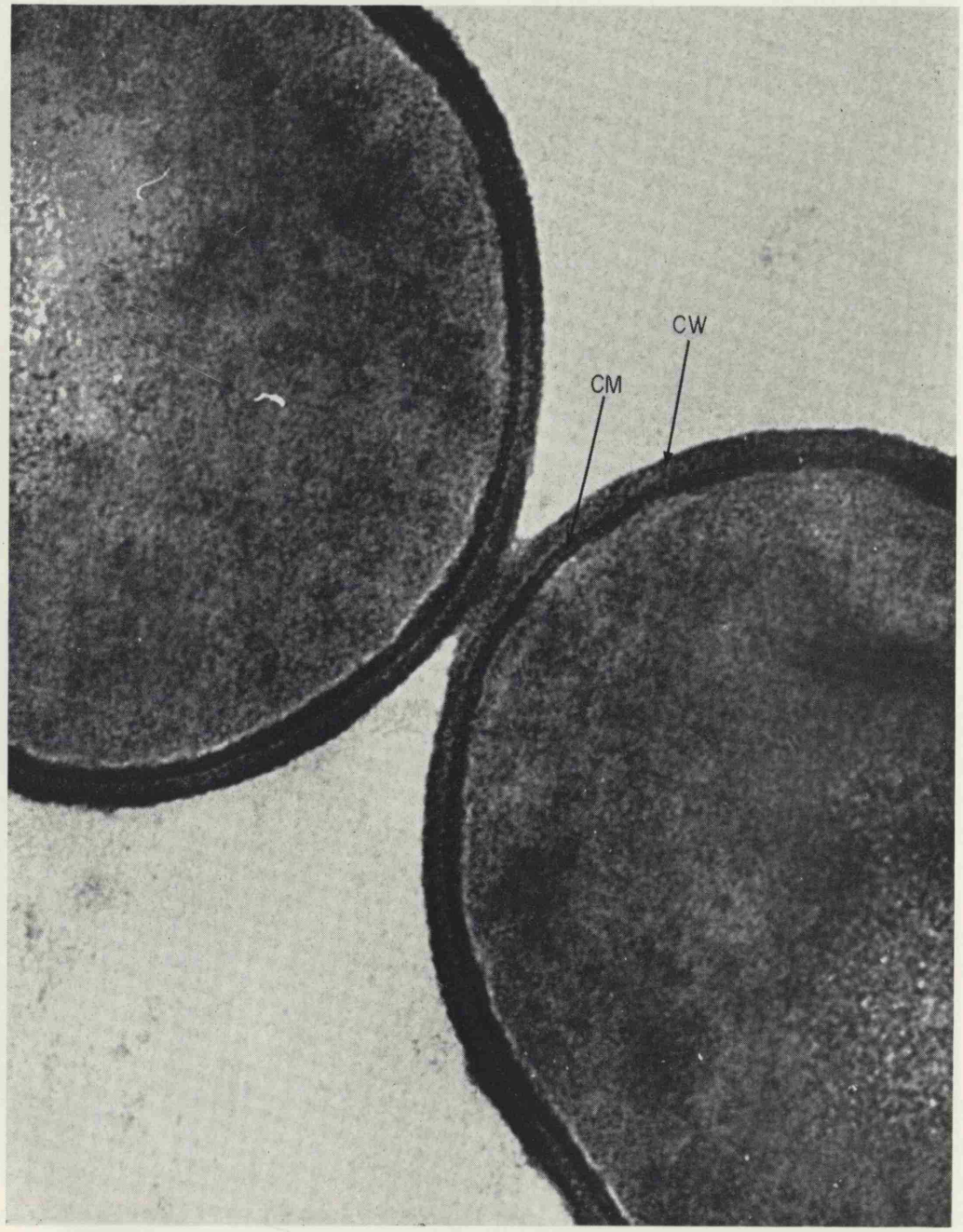


PLATE 5. Section of *M. thermosphactum* showing organization of cytoplasmic membrane (× 187,500).

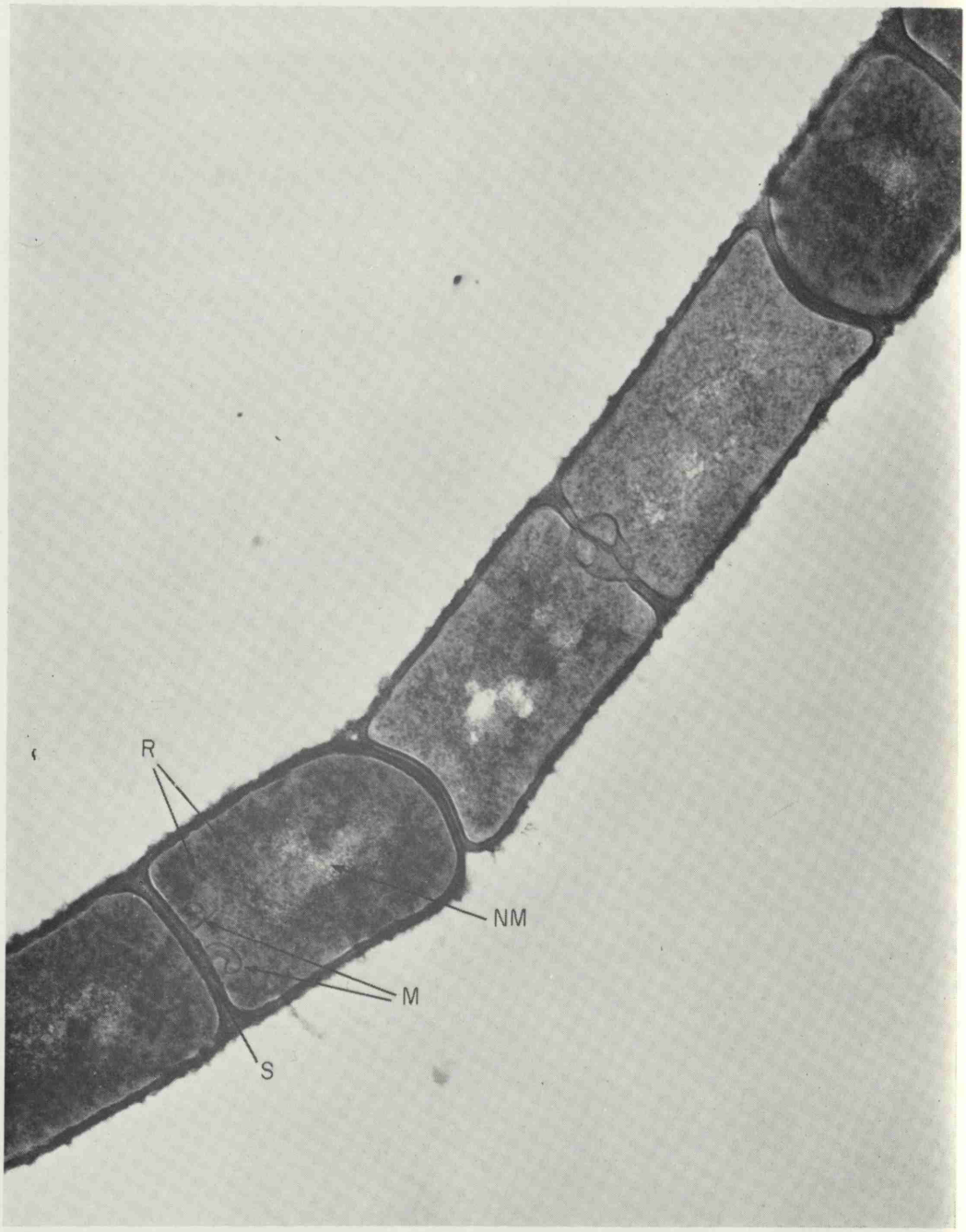


PLATE 6. Section of chain of coccobacillary form of *M. thermosphactum*. Mesosomes and cross walls are evident (× 50,000).

long kinked chains, particularly notable at 10 h. Cells at this stage were examined by electron microscopy and the long notched chains are shown in Plate 2(*b*). In this same Plate are relatively large masses of irregular rods which are possibly identical with the large bodies noted by McLean & Sulzbacher (1953). After 24 h the long chains were still evident but they then consisted of short rods and coccobacilli (Plate 1). With APT agar slide cultures, however, the reversion to short rods was not as pronounced. In liquid media the long kinked chains gave a granular turbidity, a particularly notable feature of 18–24 h cultures. On prolonged incubation the broth tended to become clear, and a heavy, slightly viscous sediment formed.

The pattern of development discussed above was common to all strains of *M. thermosphactum* examined. With *M. flavum* and *M. lacticum* only short rods were observed, and there was no evidence of long chain formation.

Thin sections of *M. thermosphactum* examined by electron microscopy showed in the central regions of the cell granular strands of nuclear material (Plates 3(*a*), 4, 6). The surrounding cytoplasm was rich in ribosomes, and contained large aggregations, thought to be (Dr. A. M. Glauert, pers. comm.) polymetaphosphate (Plate 3(*b*)) or polyhydroxybutyrate (Plate 4) inclusions. A simple form of mesosome was associated with the cytoplasmic membrane (Plates 3(*b*), 6) and the latter was bounded with the cell wall. As has been shown with other Gram positive bacteria (e.g. Edwards & Stevens, 1963), the membrane was multilayered (Plate 5). An ingrowth of the cell wall was the first obvious feature of cell division. Fully formed crosswalls were evident along the chains (Plate 6), thus providing conclusive evidence that the chains noted (Plate 1) by light microscopy consisted of individual cells. In Gram stained preparations it was not unusual to find long chains in which a few of the coccobacillary elements had failed to retain the crystal violet-iodine complex. Such cells had been noted previously by McLean & Sulzbacher (1953), Weidemann (1965) and Gardner (1966). It is noteworthy that cells of unusual appearance occurred in electron micrographs; thus in Plate 6 it can be seen that a pair of cells of comparatively low electron density were sandwiched between normal cells. The abnormal cells appeared to have weakened walls, these having been pushed in by the growth of adjacent cells. Other micrographs of the abnormal cells revealed leakage of the cytoplasm. It is conceivable that the cells discussed above are those which give an apparent Gram negative reaction.

Physiology

The properties of the organisms included in this study are summarized in Table 1. The range of temperature over which growth occurred as well as thermal resistance were the features which clearly distinguished *M. thermosphactum* from *M. lacticum* and *M. flavum*. The first mentioned organism was psychrotrophic and had low heat resistance ($D_{50}=2{\cdot}5$ min) whereas the other two grew well at 37° and had much greater resistance to heat.

All the organisms used glucose in the absence of molecular oxygen and in no instance was gas produced. Other workers (Barlow & Kitchell, 1966) were also unable to detect gas production, although McLean & Sulzbacher (1953) reported that CO_2 was formed during fermentation. The amount of acid formed was sufficient to give a positive reaction with methyl red. The products of glucose metabolism were identified

and estimated either by conventional methods or by gas chromatography. When the method of Barker & Summerson (1941) was used to estimate lactic acid, it was found that this accounted for rather more than 75% of the glucose utilized. By gas chromatography, acetic and propionic acids were detected in small amounts (equivalent to *c.* 1% of the glucose utilized). Trace amounts of *iso*butyric, *n*-butyric, *iso*valeric and *n*-valeric acids were also detected in significantly greater quantities than in the uninoculated medium.

When the collection of *M. thermosphactum* was being assembled, it was noted that the conditions of growth influenced the catalase reaction. Thus organisms grown at 20° on APT agar gave a strong positive reaction whereas those on HIA gave a weaker reaction. Incubation at 30° markedly reduced the rate of reaction so that definitely negative results were occasionally obtained. Similar results were obtained with the benzidine test of Diebel & Evans (1960). Thus, a positive reaction was given by cells grown on APT agar at 20°, whereas a negative result was often obtained with cells grown on APT agar at 30° or HIA (at 20 or 30°). The benzidine test detects iron-porphyrin compounds (Malowan, 1952), and a positive reaction with bacteria is usually assumed to be indicative of the presence of cytochromes. Low temperature spectroscopy and spectrophotometry has been used (Davidson & Hartree, 1968) to identify the cytochrome components in *M. thermosphactum*. The results (Table 1) show that this organism contains cytochromes b_1, a and a_3, and we again noted that temperature and media have a marked influence on the quantitative cytochrome content of *M. thermosphactum*. For example, cells harvested from APT broth (20°, shaking incubation) gave strong absorption at the wavelengths characteristic for the above cytochromes, whereas minimal or no absorption was found with cells grown at 30° in APT or HI broth (incubation at 20 or 30°). The reasons for differences in cytochrome and catalase content are not known. However, it does not appear to be due to the content of haem compounds in the medium. Thus in APT—the medium which favoured the formation of catalase and cytochrome—the concentrations of these substances were too low to be detected by the very sensitive haemochromogen method (Hartree, 1955).

Discussion

The observations described above have implications of practical importance. Thus, during the routine examination of isolates obtained from meat, it could be anticipated that the presence of a Gram positive coccobacilli grown under conditions which did not favour catalase formation (i.e. 30° on total count media) might lead to *M. thermosphactum* being identified with streptococci. This confusion can be avoided merely by the use of a medium (e.g. APT) and temperature (20°) which allows the organism to form easily detectable amounts of catalase. Likewise, in Gram stained preparations the long rods could be mistaken for *Kurthia* spp. However *Kurthia* are actively motile and can be easily distinguished from the nonmotile *M. thermosphactum*. In addition *Kurthia* has no demonstrable action on carbohydrates, whereas microbacteria produce enough acids to give a positive reaction with methyl red.

Among bacteria, the change from one morphological form to another is fairly well documented. With *Arthrobacter* spp., for example, Gram negative pleomorphic rods

are present in young cultures, whereas irregular masses of Gram positive cocci are dominant by 1–3 days (Conn & Dimmick, 1948). The view that this represents an obligate growth cycle may need to be modified in the light of recent observations. Thus, Ensign & Wolfe (1964) devised a simple, chemically defined medium which allowed continued growth of the coccal form. By adding various amino acids, for example, singly to the defined medium morphogenesis into the rod form was induced. It is tempting to infer that a similar situation may obtain with *M. thermosphactum* and that the sequence of events (Plate 1) observed with this organism may also be nutritionally dependent. Thus, it was noted that the morphological change from coccobacilli to rods was influenced by the medium on which the organisms were grown.

Growth conditions can also influence the catalase reaction of *M. thermosphactum*. McLean & Sulzbacher (1953) reported that older cultures occasionally failed to give a positive catalase reaction, an observation which has been confirmed by us. On certain media (e.g. HIA) even young cultures can give negative results. It is interesting to note that Weidemann (1965) also reported that choice of media influenced catalase activity of an organism thought to be *M. thermosphactum*. Thus, cultures of these organisms on nutrient agar were weakly catalase positive, whereas APT agar cultures were strongly catalase positive. Moreover, in the present study the benzidine reaction (and cytochrome content (Davidson & Hartree, 1968)) of *M. thermosphactum* was similarly affected by the growth medium. As with the catalase reaction. *M. thermosphactum* grown at 20° on APT agar always gave positive results in the benzidine test, and APT (30°) or HIA (20 or 30°) cultures were often negative. These results correlated well with the cytochrome content under different growth conditions (Davidson & Hartree, 1968). No satisfactory explanations can be offered for these disparities, although it is perhaps important to note that APT medium contains added iron (8·0 μg/ml), and it was been shown (Rawlinson & Hale, 1949) that the iron content of the growth medium influenced the cytochrome content of *Corynebacterium diphtheriae*. Furthermore (Jones, pers. comm.) found that the addition of ferric sulphate (0·02%) to a basal medium resulted in an appreciable increase in the catalase activity of *Listeria monocytogenes*.

Reports on the effects of incubation temperature on catalase production are fairly rare. Frank, Ishibashi, Reid & Ito (1963) who investigated the catalase activity of pseudomonas grown at different temperatures found that activity was greater at lower temperatures. With *M. thermosphactum* it is not known whether elevated incubation temperature directly affects synthesis of iron-porphyrin compounds, and clearly more work is necessary to establish the reasons for the observed effects.

The exact systematic position of *M. thermosphactum* also remains uncertain. Diebel & Evans (1960) and Barlow & Kitchell (1966) reported that it gave a negative result in the benzidine test, and pointed out its similarity to the lactic acid bacteria. The present authors feel that *M. thermosphactum* should be retained in the Corynebacteriaceae (rather than transferred to the Lactobacillaceae) on the basis of its positive catalase reaction, morphology and possession of functional cytochrome.

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Cytochrome as a Guide to Classifying Bacteria: Taxonomy of *Microbacterium thermosphactum*

McLean and Sulzbacher[1] proposed the name *Microbacterium thermosphactum* for a Gram-positive, catalase-positive organism that they isolated from pork sausages. At that time the genus *Microbacterium* was located in the family Lactobacteriaceae but was later transferred to the Corynebacteriaceae[2]. Deibel and Evans[3], who attached considerable taxonomic importance to the benzidine reaction, stated that while *M. flavum* and *M. lacticum* are benzidine-positive, *M. thermosphactum* is benzidine-negative and therefore more closely related to the lactic acid bacteria. Other authors have drawn attention to the uncertain taxonomy of *M. thermosphactum*[4]. The active biocatalysts for the benzidine reaction are haemoproteins and the reaction is used as a test for cytochrome in bacteria[3]. We have therefore examined *M. thermosphactum* for cytochrome and studied the cyanide-sensitivity of the NADH oxidase present in crude cell free extracts.

Table 1 gives the wavelengths of the more prominent bands (α bands) of cytochrome components that commonly occur in bacteria. We denote cytochrome components by italicized letters and the corresponding α bands by roman letters[5].

Keilin's early work[6] on cytochrome established that the pattern of cytochrome absorption bands showed greater variations among bacteria than among cells of other organisms. Studies of wide ranges of bacterial species led to classifications of bacteria in terms of (i) the effects of cyanide and carbon monoxide on their respiratory activities and (ii) the pattern of cytochrome bands[7–10].

Table 1. CYTOCHROME COMPONENTS COMMONLY OCCURRING IN BACTERIA

Nomenclature		Wavelengths (mμ) of α bands or wavelength range within which the band normally lies
Current usage	Synonyms	
a		600–604
a_1	*a'*†	590
a_2	*a''*†	632
a_3	Cytochrome oxidase	The band coincides with that of cytochrome *a*
b		562–565
b_1	*b'*†	555–560
c *		550 } These bands are normally fused to give
c_1	*e*	553 } a band at 550–552

For more complete lists see refs. 5, 9 and 10.

* The c-band in *Azotobacter vinelandii* (552) represents a mixture of two components: c_4 (551) and c_5 (555) (ref. 26).

† These symbols occur only in the very early papers on cytochrome. It is now recommended that *c'* be used to denote a variant type cytochrome *c* (ref. 5).

Cyanide-resistant respiration and an absence of cytochrome are characteristic of Lactobacillaceae[8,11]. With one probable exception[12] the genus *Clostridium* is consistently devoid of cytochrome[6,8]. Another useful taxonomic guide is the presence of cytochromes a_1 and a_2 instead of the more widespread cytochrome a. Thus the combination a_1, a_2, b_1 appears to be limited to the Enterobacteriaceae[10]. The combination a_1, a_2, b, c has been detected only among Azotobacteriaceae and one genus (*Acetobacter*) of the Pseudomonadaceae[8,10].

Most aerobic bacteria exhibit the spectrum a, b, c (less frequently a, b_1 or a, b_1, c), but earlier attempts at classification based on relative band intensities (ref. 8, for example) have proved valueless. Thus marked variations may be observed both within genera and among strains of the same species. Furthermore, the spectrum pattern may change markedly according to culture conditions (as in Table 2) and especially with degree of aeration[13]. Differences in cytochrome spectra among strains of *Corynebacterium diphtheriae* have been studied extensively because of the interesting relationship between iron nutrition, cytochrome b (or b_1) and toxin production[8,10,14–16]. The following cytochrome patterns have been recorded: $b_1 > a > c$; b (or b_1) $\gg a$; a, a_3, b, c. The band intensities are strongly influenced by culture conditions and especially by the iron content of the medium. Variations within a single strain, in terms of cytochrome bands, can be regarded as a metabolic adaptation to environment. On the other hand, mutations, both in yeasts and bacteria, can lead to deletion of a factor in the respiratory chain[16,17]: a factor which may or may not be a component of cytochrome.

Even among bacteria that show a prominent a-band representing cytochromes a and a_3, inhibition by cyanide of NADH oxidase, and of whole cell respiration, is often far from complete[7,8,16]. In the case of *Mycobacterium phlei* the incomplete inhibition by cyanide is a consequence of simultaneous functioning of two NADH oxidase systems: a cyanide-sensitive pathway (involving a_3) and a cyanide-insensitive pathway[18]. It is now established that NADH oxidase systems in Lactobacillaceae are consistently insensitive to cyanide and that the catalysts involved are flavoproteins[11].

We have examined the following strains of *M. thermosphactum:* G202 from Dr G. A. Gardner, WR7 from Dr A. G. Kitchell, H43 which was isolated at Colworth House, and NCIB 10018 which is McLean and Sulzbacher's original isolate[1]. The *M. lacticum* strain was NCIB 8541 and the *M. flavum* strain NCIB 8707. All strains were grown for 48 h on a shaker. *M. thermosphactum* was grown in APT broth[19] at 20° C or 30° C and also in heart infusion broth at 20° C. The other two species were grown in APT broth at 30° C. Cells were collected

Table 2. CYTOCHROME AND NADH OXIDASE IN *M. thermosphactum* AND IN THE OTHER TWO SPECIES OF THE GENUS *Microbacterium*[2]

	Strain	Medium	Cytochrome components in cells grown at 20° C	Cytochrome components in cells grown at 30° C	Percentage inhibition by 3 mM-HCN of NADH oxidase from cells grown at 20° C	Percentage inhibition by 3 mM-HCN of NADH oxidase from cells grown at 30° C
M. thermosphactum	NCIB 10018	APT	$a > b_1$		About 90	< 5
	G202	APT	$a < b_1$	Bands very weak or not detected	About 90	
	WR7	APT	$a < b_1$		About 90	
	H43	APT	$a < b_1$		About 90	
	NCIB 10018	Heart infusion	Very weak bands of a and b_1			
	H43	Heart infusion				
M. lacticum	NCIB 8541	APT		*a*, *b*, *c*		
M. flavum	NCIB 8707	APT		*a*, *b*, *c*		

Cytochrome bands were observed visually at room temperature in centrifugal pellets of whole cells. *M. thermosphactum* showed bands a : 602 mμ and b : 560 mμ. The other species showed a, b, c, at 602, 562, 551 mμ. In APT medium the *M. thermosphactum* strains grew rapidly at 20° C but slower at 30° C. The reverse relationship held for the other species. In the case of strain 10018 the activity of extractable NADH oxidase (on a cell weight basis) was at least 10 times higher if cells were grown at 20° C than if grown at 30° C. Thus the cyanide-resistant activity of oxidase preparations from 20° C cells was approximately equal to the total activity of equivalent preparations from 30° C cells.

by centrifuging at 20,000*g* for 15 min and washed three times with 0·03 M phosphate buffer, *p*H 7·2, in the same manner. Cell free extracts were obtained by disrupting the bacteria in a Braun cell disintegrator (Shandon) and removing cell debris by centrifuging.

NADH oxidase was measured spectrophotometrically[20], the bendizine test was carried out as described by Deibel and Evans[3], and iron was assayed with bathophenanthroline[21]. For reflectance spectrometry a Unicam SP 800 spectrophotometer was fitted with the SP 890 reflectance unit. Visual spectroscopy was carried out with a microspectroscope either at room temperature or at liquid nitrogen temperature[6,22]. For low temperature transmittance measurements a Beckman DB spectrophotometer was fitted with a liquid nitrogen reservoir which maintained the cell suspension at − 160° C (unpublished work of J. Keilin). For all observations at low temperatures cells were suspended in 50 per cent aqueous glycerol.

All strains of *M. thermosphactum* grown in APT at 20° C showed bands of cytochromes *a* and b_1 when examined at room temperature with the microspectroscope (Table 2). Spectral changes occurring in the presence of carbon monoxide showed that a_3 was present[23]. Absorption bands were faint or not detectable in *M. thermosphactum* grown in heart infusion at 20° C or in APT at 30° C. *M. flavum* and *M. lacticum* exhibited the "classical" cytochrome spectrum as seen in *Saccharomyces cerevisiae*[6]. At − 180° C the absorption bands shown by the four strains of *M. thermosphactum* were greatly sharpened. The b-band remained a single band which suggests that this species contains a single *b*-component. The d-band (β band of cytochrome b_1) was not clearly discernible: presumably it was masked by extraneous absorption. We observed good correlation between cytochrome and the benzidine reaction. Thus the four strains of *M. thermosphactum* were benzidine positive when grown on APT agar at 20° C but were negative, or only weakly positive, when grown on APT agar at 30° C or on heart infusion agar at 20° C.

Reflectance spectrometry of strains of *M. thermosphactum* revealed no peaks or inflexions that could be interpreted as cytochrome bands. Moss[24] recorded cytochrome bands in *Enterobacter aerogenes* by this technique and so we conclude that reflectance methods are unsuitable for the study of bacteria with a low cytochrome content. On the other hand, low-temperature (transmission) spectrophotometry of *M. thermosphactum* strain 10018 clearly recorded the bands a (604 mμ) and b_1 (558 mμ) but not the d-band. Although refinements of the latter technique are possible with specialized equipment[25] it is doubtful whether its sensitivity can approach that of Keilin's original simple method of visual observation

with a small-dispersion spectroscope. For success with the visual method it is essential (i) to use a concentrated suspension of cells: preferably the undiluted pellet obtained after centrifuging, and (ii) to use a powerful light source focused onto the specimen by means of the microscope condenser[22]. When the cytochrome content is very low absorption bands may be detectable only at low temperatures. When bacteria are disrupted some cytochrome may remain attached to cellular debris and part may be destroyed[26]. Therefore, while a cell free extract is more suitable for spectrophotometry, it may fail to provide a true picture of cytochrome in the intact cells.

Cultures of *M. thermosphactum* in which cytochrome bands were clearly seen yielded cell free extracts containing very active cyanide-sensitive NADH oxidase. The corresponding extract from a virtually cytochrome-free culture of *M. thermosphactum*, strain 10018 (grown at 30° C), oxidized NADH slowly, but the oxidation was insensitive to cyanide (Table 2). Strain 10018 seems to produce a small amount of a cyanide-insensitive NADH oxidase when grown in APT medium both at 20° C and at 30° C. At 20° C, which favours cytochrome formation, however, a second, more active cyanide-sensitive oxidase system is also produced (Table 2). We obtained evidence for the participation of cytochromes *a* and b_1 from spectroscopic observation of an intermittently aerated mixture of NADH and broken cells of cytochrome-containing strain 10018 (ref. 6). Thus the cyanide-sensitive NADH oxidase system of *M. thermosphactum* is unusual, being devoid of cytochrome *c*.

Our detection in *M. thermosphactum* of cytochrome and of a cyanide-sensitive NADH oxidase suggests that this organism should be retained in the family Corynebacteriaceae and not classified in the Lactobacillaceae. The basis for this conclusion is that members of the latter family do not form cytochrome. An exception to this rule, however, is the observation by Whittenbury[27] that in special conditions, in the presence of haematin, some lactic acid bacteria may produce cytochrome. There is, however, no evidence that this cytochrome is functional in the normal sense. Using the very sensitive haemochromogen test we were unable to detect haematin in either of our media. The iron contents of the APT and heart infusion media were 9·3 and 1·6 μg/ml. respectively. Comparison with work on the iron nutrition of *C. diphtheriae*[15] suggests that such a difference is relevant to the biosynthesis of cytochrome in *M. thermosphactum*.

M. thermosphactum differs from the other two species in the same genus not only on a cytochrome basis but also in terms of physiological and morphological differences[28]. Thus the precise systematic position of *M. thermosphactum* remains uncertain.

Finally, we should like to emphasize two points. (i) The observation of cytochrome in bacteria is best carried out by the visual spectroscopic technique. (ii) The application of such results to taxonomy calls for examination of a number of strains grown under a variety of conditions. Only thus can the cytochrome-synthesizing potential of a species be assessed.

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